Acta Veterinaria (Beograd), Vol. 54, No. 5-6, 411-417, 2004.

UDK 619:616.993.192.1:636.5

IMMUNOHISTOCHEMICAL INVESTIGATION OF THE *BURSA* OF *FABRICIUS* IN CHICKENS EXPERIMENTALLY INFECTED BY *EIMERIA TENELLA*

ILIĆ TAMARA, KNEŽEVIĆ MILIJANA, ALEKSIĆ-KOVAČEVIĆ SANJA, NEŠIĆ V and DIMITRIJEVIĆ SANDA

Faculty of Veterinary Medicine, Belgrade

(Received 9. May 2004)

The aim of this investigation was to detect and examine the distribution of the CD_3–T lymphocyte cell population of the Bursa Fabricius in chickens experimentally infected with Eimeria tenella. Slices of archived samples of Bursa were examined using the of direct peroxidase (DP) method. CD_3–T lymphocytes were detected with human anti-CD_3 antibody. They were localized in the cortico-medullary layer of Bursa follicles in uninfected chickens. The immunoreactivity of bursal tissue was directly correlated with the stage of development of E. tenella, with the highest intensity in the epithelium of the folds and in the layers of follicles between 3 and 5 days after the infection, predominantly in the cortico-medullary layer of the follicles. These results demonstrate the existence of an early cellular immunological response to infection with E. tenella.

Key words: Chicken coccidiosis, CD_3 lymphocytes, cellular immunity

INTRODUCTION

The *Bursa* of *Fabricius* is the primary lymphatic organ in chickens and the site of maturation of B lymphocytes. It is analogous to the bone marrow in mammals (Glick, 1991). In chickens, this lympho-epithelial organ reaches the peak of development between 4 to 12 weeks of age, followed by gradual loss of lymphoid structure (atrophy). At full sexual maturity (from 15 - 24 weeks) complete bursal involution commences (Lillehoj and Trout, 1993).

The bursa is an organ of lobular structure with an interior filled with a number of epithelial folds dividing it into 16-18 primary plicas. Primary plicas are subdivided into secondary plicas filled with lymphoid follicles numbering 8.000 to 12.000 in the fully developed bursa. Primary plicas are covered with a double layer of epithelium, the first layer positioned inbetween the follicles, while the other interconnects the follicles and joints them with the medulla (Glick, 1994). This type of follicular connection with the plica epithelium allows transfer of antigens from the interior of the canal towards the medulla of the lymphoid follicle. In this manner, an antigen coming from the follicle medulla provokes clonal expansion demonstrating the role of the bursa in the immune response (Pinck *et al*., 1985).

Histologically, the lymphatic follicle consists of three parts: cortex, cortico-medullar (borderline) region and medulla (Boyd *et al*., 1987). In the follicular core, there are a large number of densely packed lymphocytes, plasma cells, histiocytes and macrophages. In the borderline region of the cortex and medulla, there is a basal membrane and a network of capillaries where epithelial cells are actively proliferating, as well as cells similar to splenic elliptic cells. They have secretory granules in the cytoplasm (Olah and Glick, 1978) and are thus denoted as bursal secretory dendritic cells (Glick and Olah, 1993). Their role is still not clarified but it is presumed that they are responsible for the differentiation of bursal B lymphocytes and behave as APCs (Antigen Presenting Cells) (Glick, 1994). In the core of the medulla, lymphoblasts and lymphocytes dominate. Lymphocytes localized in the cortex actively proliferate, while those that originate from the core are in later stages of maturation (Boyd and Ward, 1984).

In the dorsal part of plica are diffusely scattered T lymphocytes, which comprise 35% of the total lymphocyte population in the bursa (Eerola *et al*., 1987). The finding of both plasma cells and T lymphocytes in the *Bursa* of *Fabricius* show that this organ plays multiple roles in the immune system, and not only maturation of B lymphocytes (Aleksić-Kovačević *et al*., 1999).

MATERIAL AND METHODS

1. *Animals.* This study involved a total of 100 one day old broilers, type "Hybro". After a preparation period of 14 days, the broilers were divided into two subgroups of 20 and 80 birds. Subgroup Ia was the control group, while subgroup Ib, was inoculated at 14 days old with *E. tenella* culture. Using plastic probe directly placed into the crop, 2ml of inoculate containing $4x10^5$ sporulated oocysts of *E. tenella* was administered. A field type of *E. tenella* isolated from the caeca of diseased poultry with prominent clinical symptoms was used.

In the subsequent 7 days after infection, chicken health was monitored, as well as the clinical state and the mortality dynamics of all birds in the study. In the *E. tenella* infected subgroup, five birds were killed daily and detailed pathoanatomical examination made from the second to the seventh day after infection, that is from 16^{th} to 21^{st} day of the experiment.

2. *Specimen preparation.* Tissue samples of bursa were taken for immunohistochemical testing, after fixation in 99.9% methanol, dehydration in a series of alcohol rinses, clearance in xylol and insertion in paraffin. The paraffin-embedded sections of 3-5 μm were analyzed immunohistochemically, using the direct peroxidase (DP) method. This included the application of primary monoclonal antibodies in diluted solutions, rinsing, blocking of endogenous peroxidase and staining for visuelisation (Aleksić-Kovačević *et al*., 1997).

3. *Immunohistochemistry.* Using the direct peroxidase immunohisto-chemical method we labeled CD_3-T lymphocytes in the bursa of infected and control chickens. Commercial kits were used with rabbit antibodies raised against human T-CD_3 lymphocytes (DAKO EPOS, N^0 U 0026).

Tissue samples 3-5 μm thick were fixed in 99.9% methanol (48 hours), processed in xylol (2x15 min), then rehydrated in a series of alcohol dilutions

(100%, 96%, 70% and 50%) and rinsed in distilled water. After blocking the endogenous peroxidase (in 3% H_2O_2 for 5 min), the samples were thrice rinsed in TBS (Tris Buffered Saline, pH=7.5) for 5 min, and then anti-CD_3 antibodies were applied (Anti CD_3/HRP) for 60 min at room temperature, followed by three rinses with TBS for 5 min. each The control samples were treated in the same way with TBS alone.

The reaction was visualized with 0.05% DAB –H_2O_2 in 0.1 M imidazole-HCl (pH=7.1) for 10 min and hematoxylin was used for contrast staining.

Lastly, the samples were covered in Canada balsam and prepared for microscopy.

RESULTS

In the *Bursa* of *Fabricius* from the experimental chickens, we detected a distinct distribution pattern and different frequency of the population of CD_3 T lymphocytes.

CD_3–T lymphocytes were labeled with the commercial marker for human T lymphocytes such that their expression was detectable as a reddish finely granulated precipitate on membranes and in the cytoplasm of T lymphocytes. Differences in distribution were detected in this manner as a function of time after the infection.

Thus, 2 days following the infection, we detected only rare singular immunoreactive cells, in the bursa of the chickens. They were somewhat enlarged and localized in the cortico-medullary layer of the bursal follicles.

Findings observed 3 days following the infection were characterized by the presence of a more pronounced T cell population in the cortico-medullary than in the cortical layer of the bursal follicles. At specific loci in the epithelium of plica, we noticed several immunoreactive cells, while most of the epithelium was free of this type of cell. In the medullary layer most of bursal follicles, we detected no immuno-stained cells. In a minor proportion of follicles in the central region, we observed only two to three immunoreactive cells.

Four days following the infection, immunostained cells were detected in all layers of the follicles, but predominantly in the cortico-medullary and cortical layers. Significantly, a small number of these cells could be detected in the core of the follicles (ranging from three to seven). An even distribution of immunoreactive cells was also observed in the epithelium of plica. In specific follicles, the size of this cellular population in the cortex equalled the number in the reticulo-epithelial region. There were very prominent follicles where the number of immunoreactive cells exceeded the number in the cortex and in the cortico-medullary region. Follicles where CD_3 lymphocytes filled up the reticulo-epithelial region to a larger extent than other layers were predominant.

Five days following the infection, the CD_3-cellular population was predominantly localized in the cortico-medullary layer of bursal follicles. Inside specific follicles, we observed several immunoreactive cells in the cortex and the core but lower in number than in the central region of the follicles. A larger number

of immunoreactive cells, compared with the preceding day, was observed uniformly distributed along the epithelium of bursal plica (Figure 1).

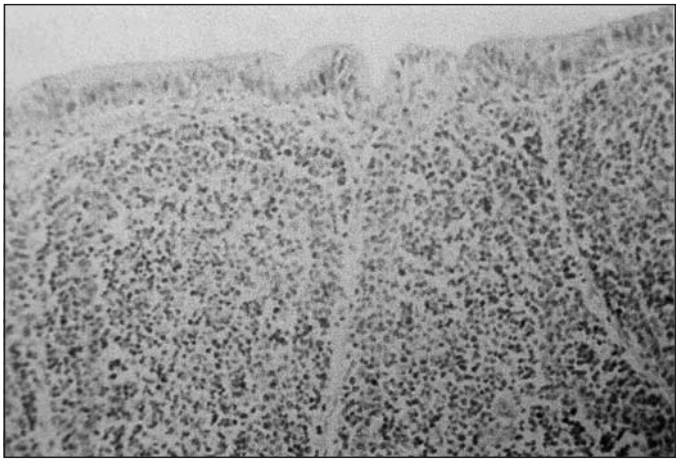

Figure 1. Chicken bursa of Fabricius, 5 days after infection showing expression of CD_3, DP, 20X

Six days following the infection, there was a reduction of the number of immunoreactive cells. The remaining cells were mostly localized in the cortico-medullary region of the follicles. In specific follicles, these cells were observed in the cortex, in equal number to the number of cells localized in the central and

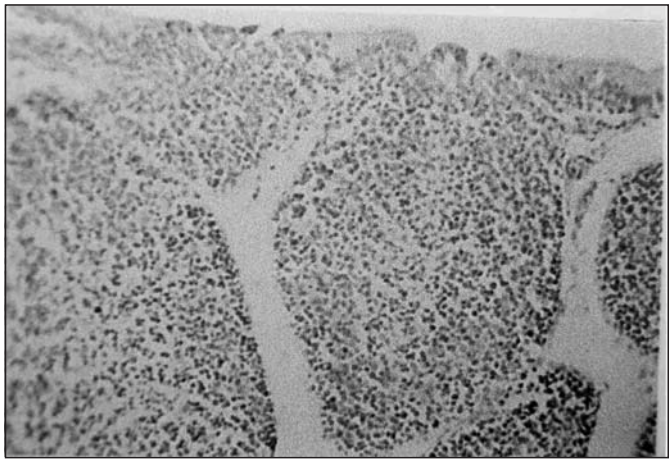

Figure 2. Chicken bursa of Fabricius, 7 days after infection showing expression of CD_3, DP, 20X

medullary region of the follicles. Immunoreactive cells were also localized in the epithelium of plica, although in smaller number than 5 days after the infection.

Seven days following the infection, we observed only a few positive cells in the epithelium of plica, a significantly lower number than previously. In the inside of the majority of follicles, immunoreactive cells were localized in the central and cortical region in a lower number than on the previous day in the same respective locations (Figure 2). We observed only two to three follicles with several labeled cells in their core.

DISCUSSION

Although all the mechanisms involved in the immune response to coccidial infection remain unknown, it has been established that the cellular response has a defensive role. In the view of the importance of CD_3-T lymphocytes in the stabilization of the antigenic receptor and the transduction activating signal through the membrane, it is possible that they have an important role in defence against coccidia (Cooper *et al*., 1991; Sharma,1997). The results obtained here support this notion.

In the bursa of healthy chickens (control), CD_3-T lymphocytes are mainly located in the cortico-medullary layer of follicles. In the experimental chickens, we also observed distribution of this subset of lymphocytes in other structures of bursa follicles depending on the stage of development of *E. tenella*.

T lymphocytes are the principal cells involved in the cellular response. Within the last few years, a number of monoclonal antibodies against the T cell receptor (TCR) and differentiation antigens have been developed that have facilitated analysis of Tcell subsets in chickens (Sharma, 2000).

Two days after experimentall infection with *E. tenella* single immunoreactive cells are localized in the cortico-medullary layer of bursa follicles, where they are normally located (Karaca *et al*., 1996).

Three and four days after infection, immunoreactive cells were present in the cortico-medullary and cortical layer of bursa follicles. On the fourth day after infection, evenly distributed CD_3 positive cells were detected in the epithelium of plica, while single CD_3-T lymphocytes were seen in the core of the follicles. Five days after infection immunoreactivity of CD_3 cells had increased, and was evenly distributed along the epithelium of the bursal plica. CD_3-T cells are predominantly located in the cortico-medullary layer, while their number is considerably lower in other layers of bursa follicles (Tanimura *et al*., 1997).

Reduction in the number of immunoreactive cells was observed in the bursa of experimentally infected chickens 6 and 7 days after the infection. At this time stained cells were mainly localized in the cortico-medullary layer of the follicles, while a somewhat lower number occurred in the cortex and the core. An insignificant number was distributed as scattered single cells in the epithelium of the bursal plica.

Birds respond to an antigenic stimulation by generating antibodies as well as by cellular immunity. Avian T cell diversity is most likely generated through

combinatorial and junctional mechanisms similar to the mechanisms that operate in mammalian T cell receptors (Reddy *et al*., 1996).

These findings document the existence of an early immunological response to infection induced by *E. tenella*. The major feature of this immune response is cellular in nature.

ACKNOWLEDGEMENT
This investigation was supported by grant 1518 from the Ministry of Science and Technology of the Republic of Serbia.

Address for correspondence:
Mr Tamara Ilić,
Faculty of Veterinary Medicine,
Bul. JNA 18, 11000 Belgrade,
Serbia&Montenegro

REFERENCES

1. *Aleksić-Kovačević S, Milosavljević P, Jovanović M, Gagić M, Knežević M,* 1997, Prednosti patohistoloških i imunohistohemijskih metoda u dijagnostici važnijih oboljenja živine, Nauka o živinarstvu, 2, 3-4, 155-60.
2. *Aleksić-Kovačević S, Knežević M, Jovanović M, Gagić M,* 1999, Distribucija antigena virusa zarazne bolesti burze (IBDV) i CD_3-T limfocita u burzama eksperimentalno inficiranih pilića, Nauka o živinarstvu, 4, 1-2, 69-73.
3. *Boyd LR, Ward HA*, 1984, Lymphoid antigenic determinants of the chicken, *Devel Comp Immunol*, 8, 149-67.
4. *Boyd LR, Wilson TJ, Mitrangas K, Ward HA*, 1987, Characterization of chicken thymic and bursal stromal cells, Alan R, Liss Inc, ed, *Avian Immunol,* 15, 29-39.
5. *Cooper MD, Chen CH, Bucy RP, Tompson CB*, 1991, Avian T cell ontogeny, *Adv Immunol*, 50, 87-117.
6. *Eerola E, Veroma T, Toivanen P*, 1987, Special features in the structural organization of the avian lymphoid system, *Avian Pathol,* 1, 9-18.
7. *Glick B,* 1991, The bursa of Fabricius and its influence on B cell development, past and present, *Vet Immunol Immunopathol*, 30, 3-12.
8. *Glick B*, 1994, The bursa of Fabricius: the evolution of a discovery, *Poultry Sci*, 73, 979-83.
9. *Glick B, Olah I,* 1993, Bursal secretory dendritic like cell: a microenvironment tissue, *Poultry Sci*, 72, 1262-6.
10. *Karaca K, Kim IJ, Reedy SK, Sharma JM,* 1996, Nitric oxide inducing factor as measure of antigen and mitogen specific T cell responses in chickens, *J Immunol Meth,* 192, 97-103.
11. *Lillehoj HS, Trout JM*, 1993, Coccidia: a review of recent advances in immunity and vaccine development, *Avian Pathol,* 22, 3-31.
12. *Olah I, Glick B,* 1978, Secretory cells in the medulla of the bursa of Fabricius, *Experientia,* 34, 1642-3.
13. *Pinck JR, Vainio O, Rajnbeek AM*, 1985, Clones of lymphocytes in individual follicles of the bursa of Fabricius, *Eur J Immunol,* 15, 83-7.
14. *Reedy SK, Suresh M, Karaca K, Sharma JM, McMillen J, Schwartz R*, 1996, Antigen-specific lymphoproliferative responses to tetanus toxoid: a means for the evaluation of Mareks disease virus-induced immunosuppression in chickens, *Vaccine*, 14, 1695-702.
15. *Tanimura N, Sharma JM*, 1997, Appearance of T cells in the bursa of Fabricius and cecal tonsils during the acute phase of infectious bursal disease virus infection in chickens, *Avian Dis,* 41, 638-45.

16. *Sharma JM*, 1997, The structure and function of the avian immune system, *Acta Vet Hung*, 45, 3, 229-38.
17. *Sharma JM, Kim IJ, Rautenschlein S, Yeh HY,* 2000, Infectious bursal disease virus of chickens: pathogenesis and immunosuppression, *Devel Comp Immunol,* 24, 223-35.

IMUNOHISTOHEMIJSKA ISPITIVANJA FABRICIJUSOVE BURZE PILIĆA EKSPERIMENTALNO INFICIRANIH SA *EIMERIA TENELLA*

ILIĆ TAMARA, KNEŽEVIĆ MILIJANA, ALEKSIĆ-KOVAČEVIĆ SANJA, NEŠIĆ V
i DIMITRIJEVIĆ SANDA

SADRŽAJ

Ispitivanja u ovom radu su imala za cilj detekciju i utvrđivanje distribucije CD_3–T limfocitne populacije u Fabricijusovoj burzi pilića veštački inficiranih sa *E. tenella*. Za izvođenje imunohistohemijskih ispitivanja korišćeni su parafinski isečci burzi, koji su nakon standardne procedure obrađeni metodom direktne peroksidaze (DP). CD_3–T limfocitna populacija detektovana humanim CD_3 antitelom, lokalizovana je u kortiko-medularnom sloju folikula burze, kod neinficiranih pilića. Imunoreaktivnost tkiva burze u direktnoj je zavisnosti od stadijuma razvoja *E. tenella*, a najintenzivnija je u epitelu plike i slojevima folikula između 3. i 5. dana posle infekcije, sa dominacijom u kortiko-medularnom sloju folikula. Rezultati dobijeni u okviru navedenih ispitivanja dokazuju postojanje ranog imunološkog odgovora na infekciju prouzrokovanu sa *E. tenella*, koji je celularnog karaktera.