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RESEARCH ARTICLE

Alteration of T, B Cells Subsets Relating to Immunosuppression and Lymphoid Depletion in Dogs with Spontaneous Acute Distemper

Yaoqian Pan[1]*, Xingyou Liu[1], Liuping Zhang[2], Yuqian Sun[1], Ruizhen Li[1], Zhike Liu[1] and Liping Meng[1]

[1]College of Animal Science, Henan Institute of Science and Technology, Xinxiang, Henan Province, 453003, China;
[2]School of Medical Science and Laboratory Medicine, Jiangsu University, Zhenjiang, Jiangsu, 212013, China
*Corresponding author: panyaoqian@163.com

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A study on dogs with spontaneous acute distemper was carried to investigate the alteration of T and B cells subsets relating to immunosuppression and lymphocytic depletion in lymphoid tissues. CD3, CD4, CD8 and CD45RO antigen expression of T cells and IgG and IgM antibody expression of B cells and distribution of canine distemper virus (CDV) antigen were detected in lymphoid tissues and bone marrow by immunohistochemistry. The results showed the CDV antigen presence in lymphocytes, follicular dendritic cells, interdigitating cells, macrophages and myelocytes. Compared to control group, a moderate loss of CD3+and CD8+ cells ($P<0.05$), significant loss of CD4+ and CD45RO+ cells ($P<0.01$) were found in T cell areas of lymphoid tissues. The ratio of CD4+/CD8+ cells was about 0.8:1. There were lots of IgG+ cells with a few scattered CD3+,CD4+and CD8+cells, few IgM+ and CD45RO+ cells in the follicle-like areas. Many IgG+ cells were detected in B cell areas, but the total of IgG+ cells were sharply reduced. The decrease orders of lymphocytic subsets were distinct in IgG+ cells and CD4+ cells, moderate in IgM+ cells and CD45RO+ cells, mild in CD8+ cells and CD3+ cells. The main reason of immunosuppression is the significant decrease of CD4 cells lead to the lower CD4/CD8 ratio, and low IgG+ cells lead to lower concentration of antibody against CDV. The reduce amount of lymphocytes especially B cells in lymphoid tissues lead to lymphoid depletion and the regeneration of immunocytes is evidently reduces in bone marrow.

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INTRODUCTION

Canine distemper virus (CDV) is a pantropic, single-stranded RNA morbillivirus and belongs to the family paramyxoviridae (Cesar *et al.*, 2011; Pan *et al*., 2013; Avila *et al*., 2014). CDV infection causes lymphopenia and immunosuppression in dogs during the early phase of disease (Wenzlow *et al*., 2007; Lee *et al*., 2010; Qeska *et al*., 2014), which is responsible for the high morbidity and mortality induced by secondary infections. In recent years, the changes of lymphoid tissue have been investigated in dogs infected with CDV experimentally or spontaneously. It has been reported that CDV-induced lesions of lymphoid tissues mainly include thymic atrophy, lymphocytes depletion in the lymphoid areas with loss of lymph nodules, no formation of secondary follicles, hyperplasia of reticular cells, focal follicular necrosis and formation of intracytoplasmic eosinophilic inclusion bodies in the cells of lymphoid tissue (Beineke *et al.*, 2009; Pawar *et al.*,2011).

In recent years, CDV antigen distribution and the changes of lymphocytic subset in lymphoid tissues of dogs with canine distemper were investigated by immunohistochemistry. Iwatsuki *et al.* (1995) and Beineke *et al.* (2009) have reported that the viral antigens located in the T cell-dependent areas and in the Thy-1-or CD4-positive cells, are also in the CD8-, CD21-, or IgM-positive cells. Therefore, Thy-1- and CD4-positive T cells serve as major target cells for CDV during the acute stage of infection. Wunschmann *et al.* (2000) have found a marked loss of CD3, CD5, CD8, IgG expression, and especially CD4 cells in lymphoid tissues with lymphocytic depletion.

To date, it has been unclear whether the changes of lymphocytic subset are related to lymphocytic depletion in

lymphoid tissue and immunosuppression of dogs with CDV. In this study, the changes of T and B cell subsets and the pathogenesis of lymphoid depletion and immunosuppression were checked by immunohisto-chemistry in dogs spontaneously infected with CDV.

MATERIALS AND METHODS

Animal selection: The 22 dogs used in this research were divided into two groups. The group 1 dogs (n=16, Nos. 1-16) were suffering from spontaneous acute canine distemper diagnosed at the basis of anti-CDV monoclonal antibody and necropsy. The group 2 (control) consisted of equal healthy Beagles of both sexes with age of 6 months (n=6, Nos. 17-22).

Tissue samples and sections collection: The tissue samples of the bone marrow, tonsil, spleen, lymph node (including mandibular and mesenteric lymph node) and aggregate nodules were collected form animals at necropsy as paraffin tissue samples and frozen tissue samples. The 4μm thick paraffin sections were cut and stained with hematoxylin and eosin. The frozen tissue samples were immerged in OCT compound (Miles Scientific, Elkhart, IN) and frozen rapidly on metal plates in liquid nitrogen. Frozen tissues were cut at 5μm with a cryostat, mounted on Superfrost Plus slides, fixed with absolute acetone for 10min, air dried at room temperature and stored at -80°C.

Immunohistochemisty: The streptavidin-biotinylated peroxidase complex (sABC) methods were performed as per manufacturers' protocol. To demonstrate CDV nucleocapsid protein, sections were immersed in citrate buffer (pH6.0) and autoclaved. For demonstration of immunoglobulin G (IgG) and IgM, sections were pretreated by proteinase K for 30 minutes at 37°C, and then washed with TBS. Sections were then blocked in 3% normal goat serum in TBS for 30 minutes at room temperature. Thereafter, sections were incubated overnight at 4°C with the primary antibodies: anti-CDV mAb (1:1000 in 3% goat serum in TBS; DAKO EPOS), rabbit anti-goat CD3 (1:160, DAKO EPOS), Anti-CD45RO/HRP, anti-canine CD4 (1:100 in PBS) and CD8(1:1000 in PBS, DAKO EPOS), rabbit anti-goat IgG and rabbit anti-goat IgM (1:800 in PBS; Kirkegaard and Perry Laboratories inc. UK). Sections were then incubated with secondary antibodies (biotinylated horse anti-mouse, biotinylated rabbit anti-rat, and biotinylated goat anti-rabbit; Vector Laboratories, Burlingame, California) for 30 min at room temperature. After visualization of the positive antigen-antibody reaction by 3,3'-diaminobenzidine (DAB: Vector Laboratory) sections were slightly counterstained with hematoxylin.

Scoring and statistical analysis: The degree of depletion in lymphoid tissues was evaluated semi quantitatively as follows: 4=normal tissue; 3=mild; 2=moderate and 1=severe depletion. Immunohistochemical determination of the expression on lymphoid cells was evaluated semi quantitatively, as follows: 0=no; 1=few; 2=some; 3=numerous positive cells; 4=nearly all cells positive. In each organ 10 randomly selected areas of each compartment were evaluated at high power by light microscopy. In order to compare groups, the SPSS17 statistical analysis software was applied. A nominal significance levels of $P<0.05$ was performed in all cases.

RESULTS

Histopathologic changes

Control dogs: There were no microscopic lesion and distribution of CDV antigen in spleen, lymph nodes, bone marrow, tonsil, and aggregate nodules of control dogs.

CDV infected dogs: Compared with the control animals, the common pathologic changes in lymphoid tissues of infected dogs, with exception to bone marrow, lymphocytes were markedly depleted, no secondary lymphoid follicles were formed, germinal center was not clearly detected, reticular cell hyperplasia showed focal or diffuse sheets in all lymphoid tissues. Some eosinophilic inclusion bodies were found in cytoplasm and nucleus of lymphocytes, follicular dendritic cells and macrophages. In addition, each lymphoid organ had particular changes. In lymph nodes, the border of cortex and paracortical areas was not clear following the formation of follicle-like areas (Fig.1A). Residual lymphocytes were often scattered in atrophic cortex ($P<0.01$). Few lymphocytes were in subcapsular sinuses. In the spleen, white pulps showed distinct atrophy and the numbers of lymphocytes decreased ($P<0.05$). Tonsils clearly lacked the discernible primary lymph follicles. Some focal small lymphocytes, resemble follicular remnants were observed in lymphoid tissues of tonsil ($P<0.01$). In the aggregate nodules, both discrete and continuous lymph tissues (solitary and aggregated lymphatic follicles) were atrophied. A few of lymphocytes were scattered in the lamina propria of villi ($P<0.05$). In bone marrow, many neutrophilic and monocytic metamyelocytes were detected. Some eosinophilic inclusion bodies were found in cytoplasm of lymphocytes, monocytes and neutrophilic promyelocytes and myelocytes (Fig. 1B).

CDV antigen was detected in all lymphoid tissues of infected dogs, especially in nucleus and cytoplasm of lymphocytes, follicular dendritic cells, interdigitating cells, macrophages and promyelocytes. CDV antigen was not observed in proliferating reticular cells and fibroblast. There were different changes in dissimilar lymphoid tissues. In lymph nodes distinct border was not observed between the cortex and medulla, CDV antigen was located in cortex and paracortical areas, especially near the capsule and trabecula areas (Fig. 2A). In spleen, the strong positive reaction was found in the cells of white pulp, periarterial lymphatic sheaths (PALS) (Fig. 2B) and around trabecula. In tonsil, the positive signals were displayed in epithelial cells of the tonsillar mucosa and glandular epithelium. In the region of the aggregate nodules, the positive cells were among the cells infiltrated in lamina propria of villi and the mucosa epithelium (Fig. 2C). In bone marrow, the positive reactions were mainly observed in promyelocytes and myelocytes (Fig. 2D).

Various antigens and IgG+ and IgM+ cells in lymphoid tissues

Control dogs: There were a huge number of CD3 and CD4 antigens, but only a few CD8, CD45RO, IgG and IgM antigen in the paracortex, interfollicular zone and PALS of

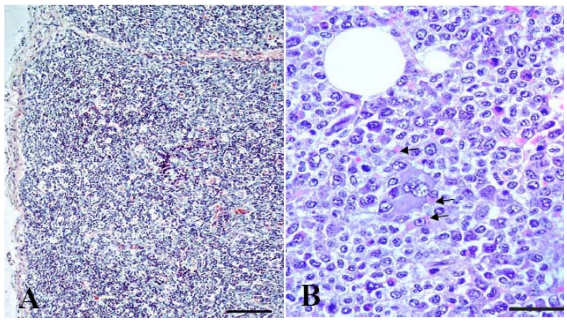

Fig.1: histopathological changes of lymph node and bone marrow. A) The border of cortex and paracortical areas is not clear following the formation of follicle-like areas. B) Some eosinophilic inclusion bodies (arrows) are present in cytoplasm of promyelocytes and myelocytes. HE staining. Bar=100μm (A) and 20μm (B).

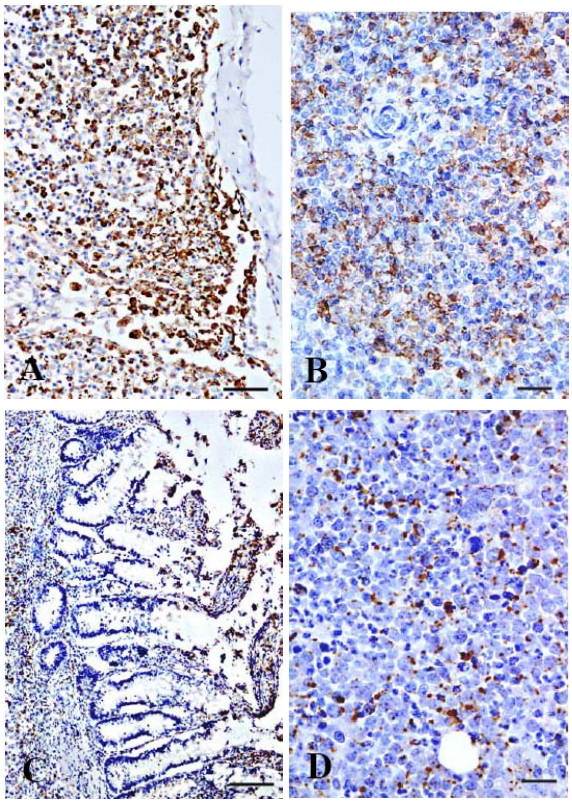

Fig. 2: Anti-CDV positive reaction in lymphoid tissues and bone marrow. A) A lot of CDV antigen positive cells in the cortex and paracortex of lymph nodes. B) A lot of CDV antigen positive cells in the white pulp and periarterial lymphoid sheaths. C) Strong positive CDV antigen expression is appeared in the cells located in lamina propria and the mucosa epithelium. D) CDV antigen positive reactions are mainly found in myelocytes of bone marrow. Anti-CDV staining. Bar=50 μm (A), 20μm (B & D) and 100μm (C).

T cell areas of lymphoid tissues. The CD4+/CD8+cell ratio was about 2:1. In B cell areas of lymphoid tissues, such as primary follicles and secondary follicles of splenic white pulp, lymph nodes, tonsil and aggregate nodules, there were a large number of IgG antigen, some of IgM, and CD4 antigen, a few CD8 and CD45RO antigen. The vast majority of lymphocytes expressed IgG, some IgM and single CD3and CD4 antigen in primary follicles and the mantle zone of secondary follicles of spleen, lymph nodes and aggregate nodules. In the dark and light zone of the

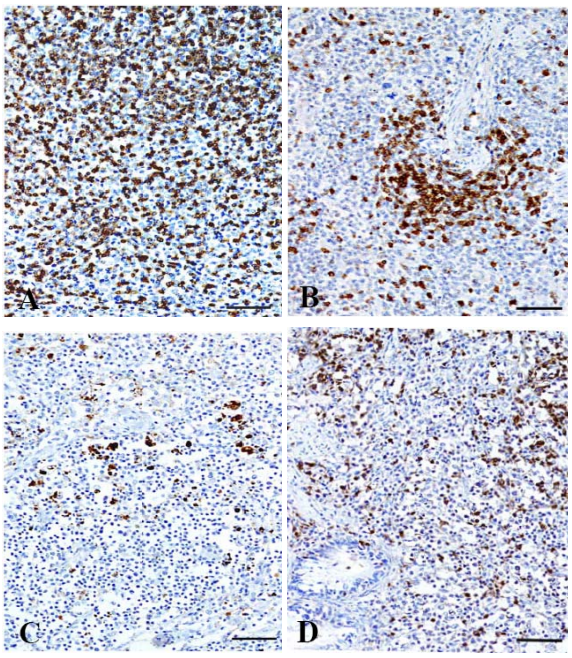

Fig. 3: Pathologic changes of lymphocytic subsets in lymphoid tissues. A) Lots of CD3+ cells mainly distributed in T cell areas. B) A lot of CD8+ cells mainly displayed around central artery in spleen. C) Many weak-staining IgG+ cells and IgG+-plasmocytes+ are detected in B cell areas and lymphoid sinus. D) Lots of CD3+ cells are observed around follicles-like areas in aggregate nodules and lamina propria. Anti-CD3 staining (A and D), anti-CD8 staining (B) and anti-IgG staining. Bar=50μm.

germinal center, weak IgG antigen reaction was detected in the majority of lymphocytes, but only weak IgM antigen expression was found in a few of lymphocytes. Some of CD4+, CD3+ and a few CD45RO+ cells were observed in the light zone. Moderate IgG+, mild IgM+, CD3 and CD4+ cells were found in bone marrow. CD3, CD4, CD8 and CD45RO expression were demonstrated on lymphocyte surface membranes. In addition, IgG and IgM antigen showed in some lymphocyte surface membranes and the cytoplasm of plasma cells. Some of lymphocytes and the large round cells (resembling macrophages) found at the cortico-medullary junction were stained by anti-CD4 mAb.

CDV infected dogs: Compared to controls a moderate loss of CD3+ (Fig.3A) and CD4+cells, significant loss of CD8+ cells ($P<0.05$) was found in T cell areas of lymphoid tissues. The CD4+/CD8+cell ratio was about 0.8:1. Few of IgG+ and IgM+ cells was observed in this zone. Some of macrophages located in the border on medullary sinus and diffuse lymphoid tissues were stained by CD45RO antibody. In spleen more CD3+ and CD8+ cells (Fig.3B), a few of CD4+ and CD45RO+ cells were mainly displayed in PALS, while some of them were scattered in splenic cord and white pulp-like areas. CD3+ cells were more than CD4+ and CD8+ cells in PALS. More CD3+ and CD4+ cells, few CD8+ cells were presented in bone marrow. In B cells areas of lymphoid tissues the follicles become the follicle-like areas. There were lots of IgG+ cells with a few of scattered CD3+,CD4+and CD8+cells, few IgM+ and CD45RO+ cells in the follicle-like areas. Many weak-staining IgG+ cells and IgG+-plasmocytes were detected in B cell areas (Fig. 3C), but the total of IgG+ cells was sharply reduced compare to controls ($P<0.01$). Some

plasma cells in medullary cord showed IgG+ or IgM+ positive reaction, respectively. CD3 and CD4 expression, except of the CD8 antigen were reduced in B cell areas compared to the control dogs ($P<0.05$). There was loss of follicles or follicle-like areas in three infected dogs. In spleen, some of IgG+, IgM+ cells were scatted in splenic cord and splenic sinus, especially along the trabecula. In aggregate nodules and tonsil, more IgG+, IgM+ and CD3+ cells (Fig.3D), a few CD4+ and CD8+ cells were detected in lamina propria of villi. A few IgG+ cells were found, but IgM+ cells were not observed in bone marrow.

DISCUSSION

Immunosuppression relating to T and B cell subsets: CDV-infected dogs usually have some special symptoms, such as leukopenia, lymphopenia, hypoproteinemia etc., which have been proved to have association with immunosuppression by numerous studies on the spontaneous or experimental cases. Beineke *et al.* (2009) reported that CDV is a lymphotrophic and highly immunosuppressive infectious agent and can causes a long lasting and profound inhibition and impairment of cellular and humoral immune functions characterized by immunosuppression, lymphocyte loss, and leucopenia. Though immunosuppression caused by CDV has been reported by many researchers, the mechanisms of immunosuppression are not too clear. According to our study the alteration of T and B cells subsets played an important role in the immunosuppression caused by CDV. It is known that CD4+ cell expresses Th cells, CD8+ cell represents Ts cells, CD45RO+ cell is relative to Tm cells, and normal CD4/CD8 ratio is responsible for humoral immune response. In present study, we found that in infected-CDV dogs the loss of CD4+ cells and CD45RO cells were more prominent than the loss of CD8+cells.The CD4+/CD8+cell ration (0.8:1) of dogs infected by CDV was lower than one of control dogs (1:1). How much is the CD4+/CD8+cell ratio in the normal dog? The ratio of 1.87:1, 1.6:1 and 3:1 was reported by Byrne (2000), Jin (2004) and Wunschmann (2000), respectively. Therefore, lower CD4+/CD8+cell ratio led to significant decrease of IgG cells and almost loss of IgM+ cells in lymphoid tissues of dogs with acute canine distemper. The diminution of IgG+ cells and IgM+ cells gave rise to the lower concentration of antibody against CDV. Lots of previous studies proved that the lower concentration of antibody against CDV is important reason to cause the immunosuppression. Steven *et al.* (1980) investigated hematologic changes of experimental animals and found that infected gnotobiotic dogs tended to have severe leucopenia because of lymphopenia. Decreased amount of serum protein was due to decreased concentrations of immunoglobulins. IgG and IgM values were too lower to be detected in fatally infected animals by Ig standards test. It has been very well known that IgG cells were the main component of B cells. IgG+ cells originated from IgG cells. The neutralization antibodies in blood, which could protect body from infection, were produced by IgG+ cells. Then, IgG cells depletion in lymphoid tissues was the basis to develop immunosuppression.

In general, the main reason of immunosuppression is that significantly decreased CD4 cells lead to the lower CD4/CD8 ratio and significantly reduced IgG+ cells arouse the lower concentration of antibody against to CDV. Additionally, decreased CD45RO+ cells and IgM+ cells may be prompt the development of immuno-suppression.

Depletion of lymphoid tissues relating to T and B cell subsets: The depletion of lymphocytes, absence of secondary follicles, loss of primary follicles and formation of follicle-like areas in lymphoid tissues of dogs with CDV has been widely reported, but the reason that caused depletion of lymphoid tissues was still too unclear. Iwatsuki *et al.* (1995) reported that the diminished immune function in the early phase of the disease is associated with viremia and partially a consequence of lysis of lymphocytes. Although different lymphocyte subsets, such as T and B cells as well as macrophages become infected, CD4+lymphocytes are most frequently affected by CDV during the acute disease phase (Iwatsuki *et al.*, 1995). Accordingly, the loss of CD4+ cells in lymphoid organs prevail the depletion of CD8+ cells (Wunschmann *et al.*, 2000). During the acute phase of distemper, lymphopenia is characterized by a transient depletion of CD4+ helper T, CD8+ cytotoxic T and CD21+ B cells from the peripheral blood (Bismarck *et al.*, 2012). Reduced numbers of circulating immune cells in CDV might be a sequel of an impaired cellular output from primary and secondary lymphoid organs (Beineke *et al.*, 2009).

In this study, CDV antigen and some of intracytoplasmic or intranuclear eosinophilic inclusion bodies were detected in lots of T and B cells of lymphoid tissues and promyelocytes or myelocytes in bone marrow. Almost all of cells relating to immune were moderately (e.g. follicular dendritic cells and interdigitating cells and promyelocytes in bone marrow.) or strongly (lymphocytes in lymphoid tissues) decrease. Because lots of immune cells were infected by CDV the severe depletion of lymphoid tissues was happened in lymph nodes, spleen, tonsil, aggregate nodules and so on. Immunohisto-chemically, compared to controls a moderate loss of CD3+, and CD8+ cells, significant loss of CD4+ and CD45RO+ cells ($P<0.01$) were found in T cell areas of lymphoid tissues, but in B cell areas the total of IgG+ cells were sharply reduced. Taking one with another, The decreased orders of lymphocytic subsets were markedly in IgG+ cells and CD4+ cells, morderate in IgM+ cells and CD45RO+ cells, mild in CD8+ cells and CD3+ cells. CD3 cells represent total T cells and a great number of them were observed in lymphoid tissues. In a word, because amount of lymphocytes, especially B cells were reduced, the elimination of secondary follicles, loss of primary follicle and formation of follicle-like areas were appeared in the lymphoid tissues and leaded to markedly lymphoid depletion in dogs with CDV.

In addition, bone marrow also related to the depletion of lymphoid tissues. In previous study, histological examination of the bone marrow of CDV infected dogs revealed no or only non-specific changes, such as hypercellularity and granulocytic hyperplasia (Breuer *et al.*, 1998) or necrosis of hematopoietic cells (Baumgartner *et al.*, 1995). In our study a lot of impairment alterations were detected in bone marrow, such as intracytoplasmic and intranuclear inclusion bodies in promyelocytes and myelocytes strongly stained by anti-CDV antibody and

almost disappearing about IgG+, IgM+ CD3+ and CD4+ cells. Therefore, because of lesions of bone marrow, the regeneration of immunocytes, such as lymphocytes and monocytes and so on, was evidently reduced, becoming an important reason of depletion of lymphoid tissues in dogs with CDV.

Conclusion: It is considered that the main reason of immunosuppression is that significantly decreased CD4 cells lead to the lower CD4/CD8 ratio and significantly reduced IgG+ cells arouse the lower concentration of antibody against to CDV. The main cause of lymphoid depletion is that a mount of lymphocytes, especially B cells were reduced and the dysfunction of regeneration of immunocytes, such as lymphocytes and monocytes and so on, evidently appears in bone marrow.

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