Ki67 expression in the cerebellum of dogs with distemper

M. YARIM[1]*, M.Y. GULBAHAR[1], T. GUVENC[1], Y.B. KABAK[1], M.O. KARAYIGIT[1]

[1]*Department of Pathology, Faculty of Veterinary Medicine, University of Ondokuz Mayis, 55139 Samsun, TURKEY.*

**Corresponding author: myarim@omu.edu.tr*

SUMMARY

As the canine distemper virus (CDV) induces demyelination in the central nervous system, partially due to losses of glial cells, the aim of the present study was to determine the Ki67 immunoreactivity in demyelinated regions from cerebellum of dogs with acute and chronic CDV infection. Cerebella from naturally infected dogs (n = 12) were routinely processed for histopathology and the observed lesions were conventionally classified as acute (n = 6) or chronic (n = 6) cases. Immunohistochemical analyses were performed in parallel for detecting the CDV antigen using a mouse anti-CDV monoclonal antibody and for evaluating the proliferation index using a rabbit anti-Ki67 polyclonal antibody. Histological and immunohistochemical findings were compared to healthy cerebellum controls (n = 6). Contrary to controls, cerebella from infected dogs exhibited demyelinated areas, often moderate to severe, in which the viral presence was confirmed. Regarding the demyelination percentage, no significant difference was found between acute and chronic cases. The glial Ki67 proliferation indexes were roughly similar in the 2 groups of infected dogs and were markedly higher than in controls whereas the mean Ki67 immunopositive glial cell counts were dramatically increased in chronically infected dogs compared to the controls or to the acutely affected dogs. These results show that the glial cell proliferation progresses with the same intensity in both acute and chronic cases, although the total number of Ki67 positive cells is higher in chronically infected dogs, suggesting that compensatory mechanisms for counteracting demyelination.

Keywords: Canine Distemper virus, dog, natural infection, demyelination, Ki67, proliferation index, glial cells.

RÉSUMÉ

Expression de Ki67 dans le cervelet de chiens atteints de la maladie de Carré

Vu que le virus de la maladie de Carré (VMC) induit une démyélinisation du système nerveux central en partie liée à la perte de cellules gliales, l'objectif de cette étude a été d'évaluer l'expression de Ki67 dans des régions démyélinisées du cervelet de chiens infectés de façon aiguë et chronique. Les cervelets de chiens naturellement infectés (n = 12) ont été analysés conventionnellement par histologie et les lésions observées ont été classées en lésions aiguës (n = 6) et chroniques (n = 6). Des analyses immunohistochimiques ont été effectuées en parallèle afin de détecter la présence des antigènes viraux en utilisant un anticorps monoclonal de souris anti-virus de la maladie de Carré et de déterminer un index de prolifération cellulaire en utilisant un anticorps polyclonal de lapin anti-Ki67. Les données histologiques et immunohistochimiques ont été comparées avec celles obtenues sur 6 cervelets issus de chiens sains. Contrairement aux contrôles, les cervelets des chiens infectés ont présenté d'importantes zones démyélinisées dans lesquelles la présence du virus a été confirmée. En ce qui concerne le pourcentage de démyélinisation, aucune différence significative n'a été trouvée entre les cas aigus et chroniques. Les index de prolifération Ki67 des cellules gliales calculés chez les chiens atteints de façon chronique sont restés identiques à ceux obtenus chez les chiens ayant une infection aiguë mais dans les 2 cas, ils ont été nettement plus élevés que chez les contrôles. En revanche, le nombre total de cellules gliales immuno-positives pour Ki67 a été considérablement augmenté dans les cas chroniques comparé aux cas aigus et aux contrôles. Ces résultats montrent que la prolifération des cellules gliales s'est déroulée avec la même intensité dans les cas chroniques et aigus bien que le nombre total de cellules positives pour Ki67 fût nettement augmenté lors d'une situation chronique, et suggèrent la mise en place de mécanismes tendant à compenser la démyélinisation.

Mots clés : Virus de la maladie de Carré, chien, infection naturelle, démyélinisation, Ki67, index de prolifération, cellules gliales.

Introduction

Canine distemper virus (CDV) may induce multifocal demyelination in the central nervous system of dogs [5, 17, 19]. The demyelinating lesions in CDV infection are mainly localized in the cerebellum and cerebellar peduncle [5, 6]. The CDV infection is often considered as a model for multiple sclerosis in humans [20].

The pathogenesis of demyelination has not been fully clarified. The initial demyelinating lesion is directly induced by virus during the active, non-inflammatory stage and the severity of myelin destruction is related to the amount of CDV antigen [21]. There are prominent astrocytosis and restricted astrogliosis in CDV infection [2, 7]. MUTINELLI *et al.* [9] reported that the presence of viral antigen in astrocytes was extensive in the astrocyte population within the demyelinating lesion (almost 65% of all astrocytes) and astrocytes were the target cells for CDV. Other authors have also identified the prominent role played by astrocytes in CDV infection [11, 18, 21, 23]. The mechanism of the demyelination was directly related to the CDV infection of oligodendrocytes, but there is little evidence for oligodendroglia infection [24, 25].

During the chronic inflammatory stage of the CDV infection, interaction of anti-viral antibodies with macrophages in a bystander mechanism are mostly responsible for the expansion of demyelination; however, initiation and perpetuation of lesions

during the acute non-inflammatory stage is directly induced by viral invasion of glial cells [21]. Evidently, virus-induced microglia cell activation plays a key role especially during the initial stages of CDV infection [16, 20].

Ki67 antigen is the prototypic cell cycle related nuclear protein, expressed by proliferating cells in all phases of the active cell cycle (G1, S, G2 and M phase) [4, 13]. It is absent in resting (G0) cells [3, 4]. Ki67 is routinely used as a neuronal marker of cell cycling and proliferation. The apoptotic profile in CNS resulting from the CDV infection has been studied [8, 14], but cell proliferation, which can be considered as one of the compensatory mechanisms of the CNS to viral invasion, has not been fully investigated in the CDV infection. The purpose of the present study was to depict a Ki67 proliferation index and to quantify Ki67 immunopositive glial cell number in demyelination areas of the cerebellum in naturally CDV infected dogs.

Materials and Methods

ANIMALS

A total of 12 dogs with spontaneous CDV infection confirmed by histopathology were included in this study. Among them, 6 exhibited an acute infection and 6 were chronically affected.

Euthanasia was performed with ketamine (1 mg/kg) / xylazine (10 mg/kg) anaesthesia followed by potassium chloride intravenous injection (2 mmol/kg). The cerebella were immediately collected and routinely processed for histopathological and immunohistochemical analyses. For control purposes, healthy canine cerebella (n = 6) were also included.

HISTOPATHOLOGICAL ANALYSIS

The cerebella embedded in paraffin were sectioned at a thickness of 5 µm and stained with haematoxylin-eosin and luxol fast-blue. Following initial diagnosis of CDV infection in the cerebellum, CDV infections were further classified as acute or chronic as described previously [18]. Briefly, acute lesions included variable degrees of demyelination without inflammatory cell infiltrations. Thick perivascular mononuclear cuffs and a moth-eaten appearance in the white matter along with a higher number of macrophages characterized the chronic lesions.

The severity of demyelination was assessed using a microscopy image analysis system (Bs200Pro Image analysis system, BAB software, Ankara, Turkey). The luxol fast blue stained cerebellum sections were observed under as stereomicroscope. The demyelination ratio was estimated by calculating the ratio of the demyelinated areas to the total cerebellar white matter area.

Upon histopathological diagnosis, additional sections were processed for immunohistochemical confirmation of the CDV antigen presence and for Ki67 expression in acute and chronic cases as well as in controls.

IMMUNOHISTOCHEMISTRY

A streptavidin-biotin detection system (Zymed Histostain Plus Bulk Kit. cat. no. 85-9043. San Francisco. CA) was used for the demonstration of CDV antigen and Ki67. The sections were dried overnight at 37°C and dewaxed in two changes of xylene for 10 minutes each, rehydrated in 100%, 95% and 70% alcohol and placed in distilled water for 10 minutes. All steps were performed in a humidified chamber at room temperature. The sections were boiled with antigen retrieval solution (Dako; cat. no. S1699, Glostrup Denmark) during 20 minutes for allowing CDV antigen staining. Endogenous peroxidase activity was quenched in 3% H_2O_2. Following protein blocking for 10 minutes, sections were incubated with either a mouse anti-CDV monoclonal antibody (1:256), (Serotec; cat. no. MCA2592. Oxford. UK) or rabbit anti-Ki67 polyclonal antibody (1:128) (Chemicon Millipore; cat. no.ab-9260. CA. USA) for 50 minutes at room temperature. The sections were then incubated with either an anti-mouse biotinylated polyvalent secondary antibody and/or an anti-rabbit secondary antibody for 10 minutes followed by the addition of the horseradish peroxidase enzyme for 10 minutes and then 3-Amino-9-ethylcarbazole (AEC) in H_2O_2 chromogene for 10-15 minutes (controlled by visual observation with a microscope). Sections were counterstained with Mayer's haematoxylin, rinsed with distilled water, and mounted with aqueous mounting medium. The distribution of immunoreactive cells was examined with a Nicon Eclipse E600 microscope.

The Ki67 glial proliferation index and the Ki67 immunopositive glial cell number in the cerebellar white matter was assessed using a microscopy image analysis system (Bs200Pro Image analysis system, BAB software, Ankara, Turkey) in 10 randomly selected demyelinated areas at X 400 magnification. The Ki67 glial proliferation index was calculated by taking the ratio of the number of positively stained cells to the total glial cell number. The slides were evaluated by two independent observers. Acute and chronic CDV cases were compared to control dogs for the Ki67 proliferation index and for the number of Ki67 immunopositive glial cells.

STATISTICAL ANALYSIS

The Mann-Whitney *U*-test was used to determine significant differences between percentages of demyelination in the *substantia alba* calculated in the acute and chronic groups. One-way analysis of variance (ANOVA) was used to reveal significant difference in glial Ki67 proliferation index and glial Ki67 immunopositive cell count among control, acute and chronic groups, following by the use of the Duncan's multiple range test [12] for multiple comparisons. Data are expressed as means ± standard deviations. Results were considered as significant when *P* values were less than 0.05.

Results

The presence of CDV infection was confirmed on the basis of histopathology and immunohistochemical localization of

CDV antigen in glial cells of the white matter. Acute and chronic cases were classified based on the histopathological evaluation.

The lesions varied from sparse foci of demyelination to widespread demyelination to a ''moth eaten'' appearance in the white matter. Astrocytosis, astrogliosis and microglia proliferation were seen in demyelinating areas and inclusion bodies were consistently present in cell nuclei. Gemistocytic astrocytes, i.e. round to oval astrocyte cells with abundant cytoplasm containing glial filaments and an eccentric nucleus or sometimes 2 nuclei, and gitter cells, i.e. microglial phagocytic cells laden with degenerating myelin were often observed. In chronic lesions, perivascular mononuclear cell infiltrations were prominent.

As shown in Table I, no demyelinated area was evidenced in the cerebellar white matter from the control dogs, whereas the demyelination percentages were moderately to greatly high in spontaneous CDV infected dogs, varying from 3.5% to 58.7%. Nevertheless, the mean demyelination percentages, 37.2% in the acute form and 36.8% in the chronic infection, have not significantly differed according to the duration of the viral infection. However, this parameter has greatly fluctuated in the group of acutely infected dogs, ranging from 3.5% (case 1) to 58.7% (case 2) whereas it has remained relatively constant in the chronically infected dogs (extreme values: 33.9% - 42.6%).

Groups	Demyelination percentage (%)
Acute CDV infection	
Case 1	3.5
Case 2	58.7
Case 3	14.9
Case 4	54.6
Case 5	44.6
Case 6	46.7
Mean ± standard deviation	**37.2 ± 22.6**
Chronic CDV infection	
Case 1	42.6
Case 2	34.8
Case 3	33.9
Case 4	37.0
Case 5	34.4
Case 6	38.1
Mean ± standard deviation	**36.8 ± 3.8**
Control	
Case 1	0
Case 2	0
Case 3	0
Case 4	0
Case 5	0
Case 6	0
Mean ± standard deviation	0 ± 0

TABLE I: Percentage of demyelination in the cerebellar white matter in acutely (n = 6) or chronically (n = 6) naturally CDV infected dogs and in healthy controls (n = 6).

Immunohistochemistry revealed nuclear as well as cytoplasmic localisations of CDV antigen immunoreactivity in gemistocytic astrocytes, glial cells, neurons, ependymal cells and endothelial cells. CDV antigen in the nucleus and cytoplasm of astrocytes was common, viral antigen and inclusion bodies being red labelled with the chromogene (figure 1), therefore confirming the CDV infection.

The Ki67 immunoreactivity was observed in the nuclei of glial cells in the cerebellum from the naturally CDV infected dogs (figure 2) as well as from the healthy dogs but, in these later, immunopositive cells were rare and scattered throughout the white matter. The Ki67 expression was seen in all glial cells as well as in gitter cells (figure 3), gemistocytic astrocytes, endothelial cells and perivascular lymphocytes (figure 4). The glial Ki67 proliferation index was significantly

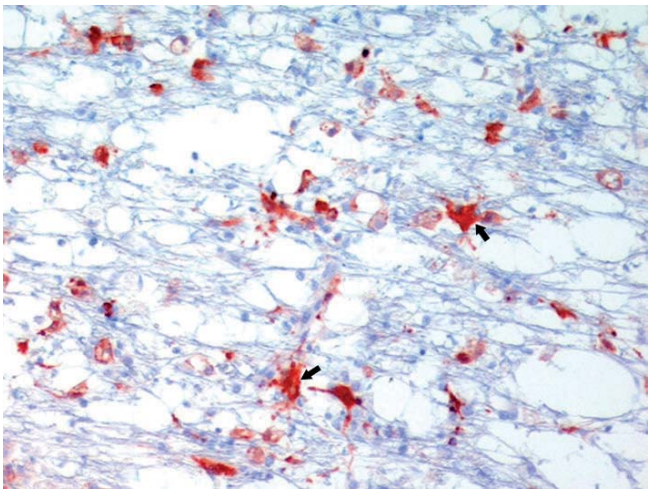

FIGURE 1: Presence of the viral CDV antigen (arrows) in demyelinating areas of the cerebellum from a spontaneous CDV infected dog. Immunohistochemistry (mouse anti-CDV monoclonal antibody), X 200.

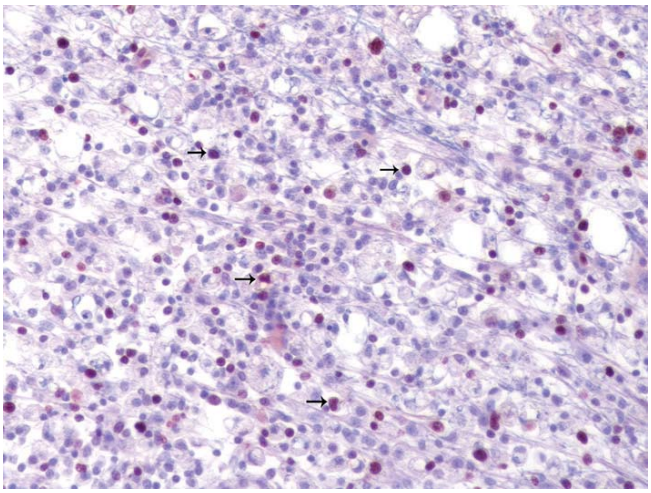

FIGURE 2: High Ki67 immunolabelling in the nuclei of the glial cells (arrows) in the cerebellar white matter of a chronically CDV infected dog. Immunohistochemistry (rabbit anti-Ki67 polyclonal antibody), X 200.

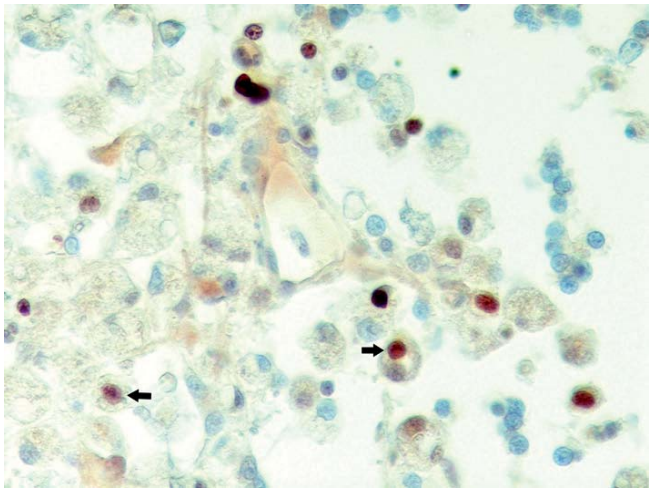

FIGURE 3: Ki67 immunolabelling in the gitter cell nuclei (arrows) in the cerebellar white matter of a chronically CDV infected dog. Immunohistochemistry (rabbit anti-Ki67 polyclonal antibody), X 400.

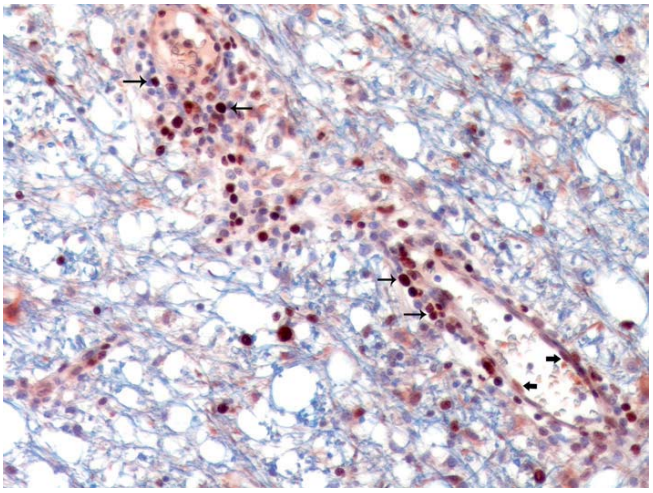

FIGURE 4: Ki67 nuclear immunolabelling of perivascular lymphocytes (thin arrows) and endothelial cells (thick arrows) in cerebellum from a chronically CDV infected dog. Immunohistochemistry (rabbit anti-Ki67 polyclonal antibody), X 200.

Groups	Glial Ki67 PI	Ki67 IGCC
Acute CDV infection		
Case 1	43.9 ± 5.4	23.6 ± 4.2
Case 2	36.0 ± 12.1	28.7 ± 7.2
Case 3	31.7 ± 9.0	37.6 ± 6.3
Case 4	27.7 ± 4.2	51.4 ± 6.3
Case 5	32.7 ± 6.6	30.8 ± 2.6
Case 6	31.6 ± 5.4	32.3 ± 4.7
Mean ± standard deviation	**33.9 ± 2.3^{b}**	**34.1 ± 9.6^{a}**
Chronic CDV infection		
Case 1	33.4 ± 5.6	63.8 ± 10.1
Case 2	27.3 ± 2.7	134.7 ± 19.0
Case 3	27.2 ± 4.2	157.8 ± 18.1
Case 4	48.0 ± 3.4	136.0 ± 13.1
Case 5	31.3 ± 2.6	117.5 ± 23.3
Case 6	38.8 ± 3.7	126.3 ± 29.4
Mean ± standard deviation	**34.3 ± 3.2^{b}**	**122.7 ± 31.8^{b}**
Control		
Case 1	16.1 ± 3.8	15.1 ± 2.6
Case 2	14.9 ± 0.7	25.5 ± 4.5
Case 3	17.2 ± 2.2	15.9 ± 3.5
Case 4	17.7 ± 1.6	15.6 ± 3.2
Case 5	16.3 ± 2.8	16.8 ± 4.9
Case 6	16.9 ± 2.2	15.8 ± 3.3
Mean ± standard deviation	**16.5 ± 0.4^{a}**	**17.5 ± 4.0^{a}**

Glial Ki67 PI: Glial Ki67 proliferation index given by the ratio of the number of immunopositive cells to the total glial cell number at X400 magnification; Ki67 IGCC: Ki67 immunopositive glial cell count on 10 microscopic fields at X400 magnification.
Different superscripts ^{a,b} in the same column evidence significant difference ($P < 0.05$) between groups.

TABLE II: Ki67 immunoreactivity (determination of the glial Ki67 proliferation index and of the Ki67 immunopositive glial cell count) in the cerebellar white matter in acutely (n = 6) or chronically (n = 6) naturally CDV infected dogs and in healthy controls (n = 6).

higher in infected CDV dogs (with acute and chronic infections) than in controls ($P < 0.05$) (Table II) but this parameter was similar between the groups of acutely and chronically infected dogs. By contrast, the number of Ki67 immunoreactive glial cells in demyelination areas was markedly higher in chronically infected cerebella compared to acutely infected and control cerebella ($P < 0.05$) (Table II); however, no statistically significant difference was found between acute and control groups ($P > 0.05$). In addition, no significant correlation was established between the ratio of demyelination and the glial Ki67 proliferation index in CDV infected dogs.

Discussion

Through this study, the Ki67 expression in glial cells in the white matter of the canine cerebellum during acute and chronic CDV infections was investigated for the first time. The glial Ki67 proliferation index was significantly higher in acute and chronic CDV infected cerebella compared to controls, depicting an increase in the number of proliferative glial cells in damaged CNS regions as a result of viral invasion [2, 10]. Moreover, although there was no significant difference in the glial Ki67 proliferation index between acute and chronic infected dogs, the numbers of Ki67 immunopositive glial cells in the cerebellar white matter were dramatically increased in chronically infected dogs compared to the acutely infected ones. The hallmark of the present study is that the number of Ki67 positive cells was greatly enhanced in chronic cases despite presence of severe demyelinated areas. Among the Ki67 positive cell populations found in the cerebellar white matter in chronic cases, the oligodendrocytes may probably be abundant. As it was recently discussed, the oligodendrocyte precursor cells are considered to be in a high proportion among the Ki67 positive oligodendrocytes in demyelinated regions [10, 15, 25]. However, in severe demyelinated regions, these precursors do not acquire the capacity to synthesize myelin because the astrocyte progenitors are not able to synthesize and release molecules such as Insulin-like growth factor-1 (IGF-1) required for oligodendrocyte differentiation, maturation, and myelin formation [1]. Consequently, although oligodendrocytes seem to be in large amount in demyelinated areas, they can not repair the degenerated areas and compensate the myelin loss [10, 15, 24] and healing in demyelinated regions cannot occur. The results in the present study suggest that the high Ki67 expression in glial cells which characterizes an increased cell proliferation may correspond to an intense assay for re-myelinisation in chronic cases.

One of the marked findings in the present study is that microglia originated gitter cells also expressing Ki67, that indicating microglia cell proliferation [16]. In agreement with that, a recent *in vitro* study suggested that activated microglia cells can promote neurogenesis from neural stem cells [22]. In this way, the microglia cell proliferation may be a part of an effort in neuronal regeneration in addition to their routine involvement in phagocytosis of degenerated tissues.

As a conclusion, the Ki67 proliferation index increases in the cerebellum of CDV infected dogs suggesting the occurrence of a compensatory effort in order to replace the cell losses due to the viral invasion by glial cell proliferation. Further studies are required to determine the type and ratio of proliferating glial cells and their roles in the repair mechanisms.

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