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IMMUNOHISTOCHEMICAL DISTRIBUTION OF ALPHA B-CRYSTALLIN IN THE CEREBELLUM OF DOGS INFECTED WITH CANINE DISTEMPER VIRUS

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The cerebella of 12 dogs infected with canine distemper virus (CDV) and those of three normal dogs were examined. The avidin-biotin-peroxidase complex technique was used to detect αB-crystallin (αB-c) immunoreactivity and immunolocalisation of the CDV antigen. CDV antigens, immunopositive astrocytes, oligodendrocytes and granular neurons were seen in both the white and grey matter of the infected dogs. In the controls, αB-c immunopositive glial cells were seen in the white matter and around the Purkinje cells. In dogs with distemper, αB-c immunoreactivity was not observed in some of the glial cells around the Purkinje cells. A significant negative correlation of $P < 0.01$ level was found between areas of severe demyelination and the number of αB-c immunopositive cells in dogs infected with CDV. Such correlation was not observed between mild and moderate demyelinating areas and αB-c immunostaining. The αB-crystallin/ total number of cells ratio was found to be significant in severely affected demyelinating areas ($P < 0.05$). These data indicate that there was a relationship between the degrees of CDV associated with demyelination and the level of αB-c expression in the glial cells.

Key words: αB-crystallin, canine distemper virus, cerebellum, dog, immunohistochemistry

CDV causes a severe immunosuppressive and neurological disease in dogs and other carnivores, which is characterised by multifocal lesions in the grey and white matter of the central nervous system (CNS) (Summers and Appel, 1994).

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The hallmark of white matter lesions is progressive demyelination, but the mechanism of demyelination in distemper is not yet understood clearly (Kabakci et al., 2004; Vandevelde and Zurbriggen, 2005). CDV infection is often considered as a model for multiple sclerosis (MS) in humans (Vandevelde and Zurbriggen, 2005).

αB-crystallin (αB-c) belongs to a family of small heat-shock proteins (HSP), and is induced in response to different stresses including heat shock, oxidative stress, metal ions and cytokines (Konishi et al., 1997; Goldbaum and Richter-Landsberg, 2001; Richter-Landsberg and Goldbaum, 2003). αB-c has chaperone-like properties, preventing aggregation of damaged or misfolded proteins induced by cell stress. It is constitutively expressed in many tissues of humans and animals, such as lens of the eye, heart, and skeletal muscles (Iwaki et al., 1992; Richter-Landsberg and Goldbaum, 2003). In the normal CNS, a low level of αB-c is present in astrocytes and oligodendrocytes, but not in neurons. Expression of αB-c is increased in different pathological conditions of the CNS, particularly in MS (Bajramovic et al., 1997; Bajramovic et al., 2000). It is also present in ballooned neurons, which are observed during various neurodegenerative diseases such as Pick's disease and Alzheimer's disease (Bajramovic et al., 1997; Head and Goldman, 2000; Dabir et al., 2004).

The aims of the present study were (1) to determine the immunohistochemical distribution of αB-c in the cerebellum of CDV-infected and noninfected dogs and (2) to determine the relationship between αB-c and demyelination in CDV infection.

Materials and methods

Animals and tissue collection

Fifteen (5 male and 10 female) dogs of various breeds were used in the study, ranging in age from 3 months to 2 years. The CDV-infected group consisted of 12 dogs infected naturally with CDV, and three noninfected dogs, which died of causes unrelated to CDV or any neurological disorder, served as a control group. Samples of cerebellar tissue collected from dogs infected with CDV and from the three negative controls were fixed in 10% neutral formalin solution. Tissue samples were then dehydrated in ethanol and were embedded in paraffin wax. Serial sections of 5 μm were cut and stained with haematoxylin and eosin for histopathological evaluation. Demyelination was categorised as mild (n = 4), moderate (n = 4), and severe (n = 4) by comparing the percentage of the area of demyelination with the total image area, and corresponded to values lower than 5%, between 5% and 10%, and more than 10%, respectively. Additional sections were processed for immunohistochemical investigation of the CDV antigen and αB-c.

Immunohistochemistry

The sections, placed on slides coated with 3-aminopropyltriethoxysilane (Sigma, St. Louis, MT, USA), were stained by the streptavidin-biotin-peroxidase complex (SBPC) technique (Zymed, Histostain Plus Kit, California, USA) using monoclonal antibodies. Mouse monoclonal antibodies included the anti-CDV antigen (MCA1893, Serotec, Oxford, UK) and the anti-αB-c (SPA-222, Stressgen, Victoria, Canada). The slides were dried overnight at 37 °C, dewaxed, changing the xylene twice, with a 10-min interval between changes, rehydrated using the graded alcohol series, and placed in distilled water for 10 min. Antigen retrieval was facilitated by heating in citrate buffer (pH 6.0) for 20 min in a microwave oven with a power of 600 W. The slides were dipped in freshly prepared absolute methanol containing hydrogen peroxide (H_2O_2) 3% v/v for 5 min to block endogenous peroxidase activity. After washing with phosphate buffer solution (PBS), all sections were preincubated in 10% normal goat serum for 30 min at room temperature (RT) to block nonspecific binding of the second-step antibody. Sections were incubated with primary antibodies for 60 min at RT and were rinsed with PBS. The sections were then incubated with broad-spectrum biotin-conjugated second-step antibody (85-9043, Zymed, California, USA) for 10 min at RT and were then rinsed in PBS. SBPC was applied for 10 min at room temperature. Amino ethyl carbazole (AEC) or 3,3'-Diaminobenzidine (DAB) was used as chromogen in H_2O_2 for 10 min (controlled by visual observation with a microscope). The sections were counterstained with Mayer's haematoxylin for 1 min, rinsed with tap water, and mounted with an aqueous mounting medium.

Positive and negative control sections of cerebellum for CDV and αB-c were used in all immunolabelling procedures. The distribution of immunoreactive cells was examined with a Nikon E-600 microscope.

Image analysis and statistics

The percentage of the total area of demyelination, the number of αB-c immunopositive cells, and the total number of cells were assessed by using a microscopy image analysis system (Bs200P Image Analysis System, BAB software, Ankara, Turkey). A total of 10 fields were chosen and analysed at ×400 magnification. Bivariate correlation test (Spearson's correlation) was used for determination of the correlation between the number of αB-c positive cells and the severity of demyelination. The ratio of αB-c positive cells to the total number of cells was estimated, and then its correlation with demyelination was statistically evaluated. One-way analysis of variance (ANOVA) was used to show a significant difference among mild, moderate and severe cases of demyelination. If significant in ANOVA, Duncan's multiple range test was used for multiple comparisons. A P value of less than 0.05 was considered significant in all statistical analyses. All data are expressed as mean ± standard deviation.

Results

Histopathological findings included variable degrees of demyelination, with or without inflammatory cell infiltrations, astrocytic gliosis, and the presence of gemistocytes and Gitter cells. Intranuclear inclusion bodies, astrocytic syncytia and thick perivascular mononuclear cuffs were also observed in some cases.

The immunolocalisation of the CDV antigen was diffuse in some sections, but focal in others, particularly localised in the affected areas (Fig. 1). CDV antigens were observed in the cytoplasm and nuclei of the cells including astrocytes, oligodendrocytes and granular neurons in both the white and grey matter.

Positive immunoreactivity of αB-c was not observed in either neurons or Purkinje cells in the cerebella of any of the dogs (Fig. 2). In the control dogs, αB-c immunopositive glial cells were seen in the white matter and around the Purkinje cells (Fig. 3). Various degrees of positive staining were detected in the cytoplasm of these cells. Some of the glial cells, localised around the Purkinje cells, were negative for αB-c immunolabelling in distemper cases (Fig. 4). The counts of αB-c positive cells in the white matter were decreased in proportion to the intensity of demyelination (Figs 5–6).

A statistically significant ($P < 0.01$) correlation was found between the areas of severe demyelination and the number of the αB-c immunopositive cells in dogs infected with CDV. However, correlation between mild and moderate demyelinating areas and αB-c immunostaining was not observed. Additionally, the αB-crystallin/total number of cells ratio was not significant between the control group and the mild/moderate demyelinating areas, but was found to be significant in severely affected areas ($P < 0.05$). The statistical data are summarised in Table 1.

Discussion

αB-c is a small HSP induced by heat shock and other stressful situations. It is expressed during several neurodegenerative disorders in humans. In this study, we examined the expression of αB-c in the cerebella of CDV-infected and noninfected dogs.

MS is a chronic and inflammatory demyelinating disease of the CNS in humans, and CDV is a model for MS (Vandevelde and Zurbriggen, 2005)**.** In MS lesions, αB-c was present in astrocytes and oligodendrocytes in areas of demyelination in the CNS and was believed to be an autoantigen (Bajramovic et al., 1997; Van Noort et al., 1998; Richter-Landsberg and Goldbaum, 2003). However, in the present study the number of αB-c positive cells decreased in areas of severe demyelination, and a significant negative correlation was also found between the percentage of the area of demyelination and αB-c positive cell count.

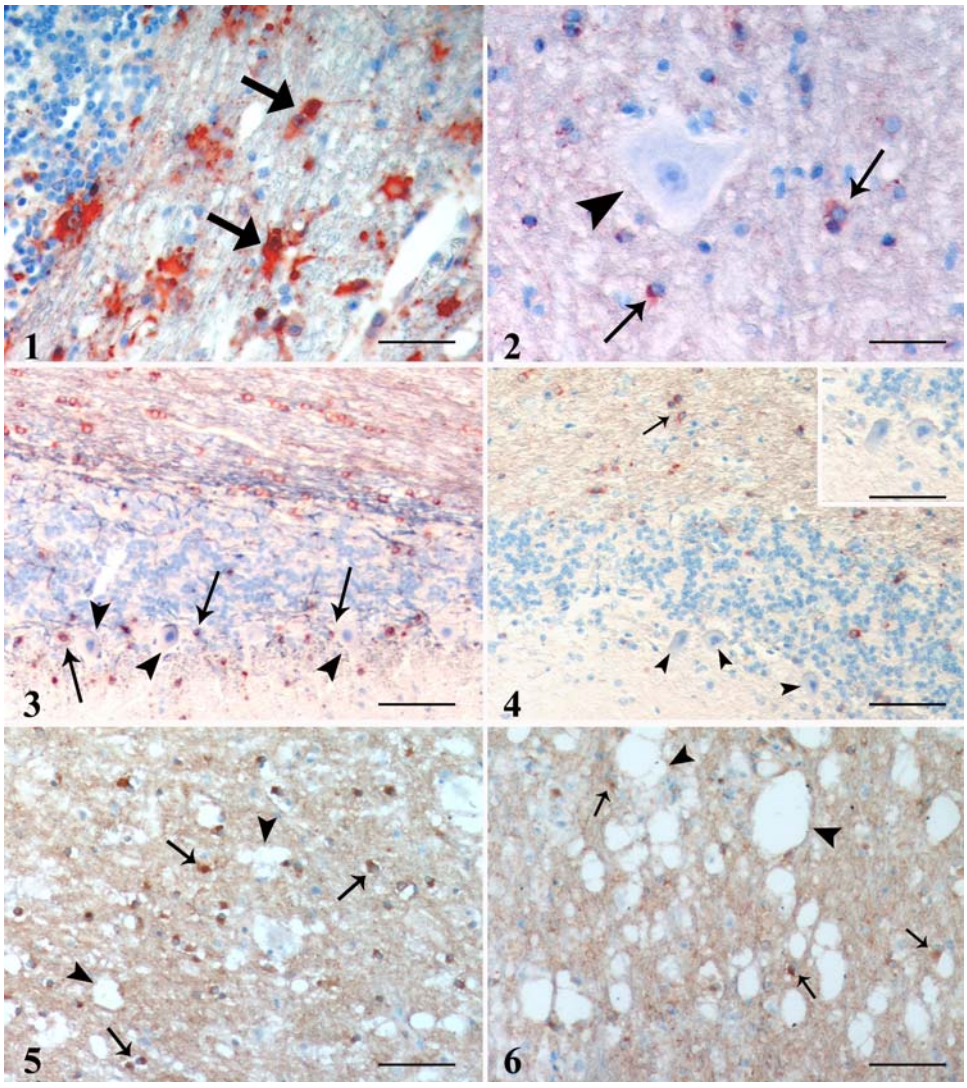

Fig. 1. Immunohistochemistry of CDV antigen in demyelinating areas of the cerebellum infected with CDV (arrows). IHC/AEC. Bar: 20 µm. *Fig. 2.* An αB-c immunonegative neuron (arrowhead) and positive glial cells (arrows). IHC/AEC. Bar: 20 µm. *Fig. 3.* Control dog. Marked αB-c immunostaining of some glial cells (arrows) around the Purkinje cells (arrowheads). IHC/AEC. Bar: 60 µm. *Fig. 4.* CDV-infected dog cerebellum. Note negative immunostaining of glial cells around the Purkinje cells (arrowheads) and positive immunostaining of those in the white matter of cerebellum (arrow) for αB-c. IHC/AEC. Bar: 60 µm. Inset shows higher magnification of area indicated by arrowheads. Bar: 40 µm. *Fig. 5.* αB-c positive glial cells (arrows) and demyelinating areas (arrowheads) in a case characterised by mild demyelination. IHC/DAB. Bar: 40 µm. *Fig. 6.* αB-c positive glial cells (arrows) and demyelinating areas (arrowheads) in a case of severe demyelination. IHC/DAB. Bar: 40 µm

Table 1

Proportion of αB-c positive cells to total cells in the CDV-infected group showing variable severity levels of demyelination and in the control group

Severity of demyelination		αB-c$^+$/total cell
Severe	n = 4	0.13 ± 0.03^a
Moderate	n = 4	0.42 ± 0.09^b
Mild	n = 4	0.47 ± 0.09^b
Control	n = 3	0.36 ± 0.06^b

Means with different superscript letters in the same column differ significantly ($P < 0.05$) by Duncan's multiple range test

This finding reflects the possibility that the pathogenesis of demyelination in canine distemper may be different from that of MS lesions. Moreover, it may be speculated from the present study that the critical point of down-regulation of αB-c was due to the percentage of the area of demyelination.

As a cytoprotective function, αB-c is upregulated in oligodendrocytes after both heat shock and oxidative stress (Goldbaum and Richter-Landsberg, 2001; Richter-Landsberg and Goldbaum, 2003). In the present study, difference in αB-c immunoreactivity was not found between mild to moderately affected areas of demyelination and control cerebella. However, the expression of αB-c was decreased in severely affected demyelinating areas. The present study reports that both the stress associated with CDV infection and the lack of αB-c immunoreactivity may trigger the progression of demyelination. Moreover, in distemper cases, αB-c immunolabelling was absent around the Purkinje cells in contrast to the cerebella of the control group. This condition may suggest that these cells were not exposed to any kind of stressful condition such as CDV infection.

Although the functional significance of αB-c is not clear, the previous reports showed that accumulation of αB-c in the neural cytoplasm may be caused by a chromatolytic response, perikaryal reaction to axonal involvement, or ischaemic stress (Kato et al., 1992; Minami et al., 2003). It was observed that the neurons of all the dogs used in this study were not immunopositive for αB-c. This finding possibly indicates that the neurons were not exposed to any stress conditions, or that αB-c did not accumulate to immunohistochemically detectable levels in CDV infection.

In conclusion, in this study a relationship was observed between the degree of demyelination and the level of αB-c expression in glial cells. CDV especially may destroy astrocytes and oligodendrocytes expressing αB-c; however, further studies are needed to elucidate other mechanisms involved in demyelination in canine distemper.

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