Abstract CD44 antigen (CD44), the principle cell surface receptor for hyaluronate, is up-regulated in the human demyelinating disease multiple sclerosis on fibrous astrocytes. As astrocytes are the main target cell of canine distemper virus (CDV), the consequences of a CDV infection on the CD44 expression and distribution in brains with spontaneous demyelinating canine distemper encephalitis (CDE) were of interest. Thirteen acute, 35 subacute, and 11 chronic plaques of nine dogs with immunohistologically confirmed CDE and brains of control dogs were included in the study. For light microscopy, 5-μm-thick serial sections were stained with H&E and incubated with monoclonal antibodies (mAbs) against CD44 and canine distemper virus nucleoprotein and polyclonal antibodies (pAbs) against glial fibrillary acidic protein (GFAP) and myelin basic protein (MBP). For immunoelectron microscopy, 90-nm-thick sections were double stained with anti-GFAP and anti-CD44 mAbs to specify CD44-expressing structures. In controls, CD44 was diffusely distributed in the white matter and single meningeal cells exhibited a marginal expression of the antigen. In acute and more prominently in subacute demyelinating encephalitis, there was a plaque-associated up-regulation of CD44 which paralleled GFAP. In chronic demyelinating lesions, a reduction of CD44 associated with a loss of GFAP-positive astrocytes was noted. Additionally, in chronic plaques, CD44 was expressed on the cell membrane of perivascular mononuclear cells. Immunoelectron microscopically, in controls, CD44 was rarely demonstrated on astrocytic cell processes. In contrast, in brains with CDE CD44 was found on the cell membrane of broadened astrocytic cell processes. In summary, CD44 is up-regulated on astrocytes in the early phase of CDE and seems to represent a marker for the activation of immune cells in the late phase of the infection.

Key words CD44 · Demyelination · Dog · Canine distemper virus infection

Introduction

Canine distemper virus (CDV) is, like measles virus (MV), a member of the genus Morbillivirus of the Paramyxoviridae family [20]. Due to the central nervous system (CNS) tropism of the virus and the associated neuropathological changes, canine distemper encephalitis (CDE) has advanced to an important model for human demyelinating diseases [7]. Demyelination in CDE is a biphasic process. Initiation of demyelination is ascribed to a direct action of the virus. As virus antigen could be readily demonstrated in astrocytes, the main target cell of the virus [17], but not in oligodendrocytes, the pathogenesis of this first phase of demyelination remained obscure. Recent studies, however, suggested, that oligodendrocytes undergo a restrictive infection with transcription of CDV RNA [29, 30]. As demyelination proceeds despite a dramatic reduction or elimination of the virus [1], the main focus of several studies was on the associated immune response to target possible underlying immunopathological mechanisms. Indeed, a striking up-regulation of the major histocompatibility complex class II antigen mainly on microglia in subacute and chronic demyelinating CDE and a T cell-dominated immune response were observed [2, 25]. A detailed immunophenotyping study of the associated cells revealed that, dependent on the age of the plaques, CD4⁺, CD8⁺ and B cells show a different spatial distribution [28]. Accordingly, CD8⁺ lymphocytes invade the brain earlier and are the dominating cell population in the neuropil, while in the perivascular space the lymphocytic infiltrate is mainly composed of CD4⁺ and B cells.

Essentially, so far, most studies investigated the participating cells but the role of the extracellular matrix (ECM)
and its receptors in the demyelination process remains unclear. However, in view of the importance of astrocytes as a major source of ECM proteins [16] and their importance for maintaining structure and relationships in the brain, the consequences of a CDV infection of this cell population on the ECM seemed to be an important question to be addressed. Asher et al. [4] demonstrated that a hyaluronate-based ECM must exist in the canine CNS as glial hyaluronate binding protein (GHAP) was demonstrated immunoelectron microscopically in the space between myelin sheaths and astrocytic processes in the canine spinal cord white matter. The brain seems to be an organ rich in hyaladherins [13] and the principal cell surface receptor for hyaluronate is the CD44 antigen (CD44). CD44 exists as a standard form (CD44S) [11, 12] and at least ten isoforms (CD44V) [23]. Human CD44 was first purified by Underhill et al. [26] and is a 85-kDa protein. It is involved in lymphocyte homing [24] and, in the normal human brain, CD44 is associated with fibrous astrocytes [10].

Canine CD44 is a 85- to 90-kDa protein expressed in lymph node tissue [15] and canine CD44-specific antibodies have been used in transplantation studies [21, 22]. A monoclonal CD44 specific antibody directed against a CD44 mRNA-expressing [3] macrophage/monocyte cell line from a dog with malignant histiocytosis [27] bound to macrophages/monocytes, subsets of lymphocytes and epithelial cells. Additionally, the antigen was expressed on normal CNS white matter [3].

Based on these data, the main interest of this study was to evaluate the expression and distribution of CD44 in non-demyelinated and demyelinated lesions in CDE.

### Materials and methods

**Tissue preparation**

The cerebella of three control dogs and animals with spontaneous acute (n = 3), subacute (n = 3), and chronic (n = 3) CDE were used in the study. Thirteen acute, 35 subacute, and 11 chronic lesions were investigated. Tissues were either fixed in 10% non-buffered formalin, and processed for routine histology (H&E stain) and immunohistochemistry, or fixed in 2% paraformaldehyde in 0.1 M PBS (pH 7.8; 650 mOsmol) for 24 h at room temperature (RT) for immunoelectron microscopy.

**Immunohistochemistry**

Serial paraffin sections of 4 μm thickness were cut and mounted on Superfrost Plus slides (Menzel Gläser, Glastbearbeitungswerk, Braunschweig, Germany). The primary antibodies used in this study were: polyclonal antibody (pAb) against glial fibrillary acidic protein (GFAP; Dako, Hamburg, Germany; Z0334, 1:500); anti-myelin basic protein (MBP) pAb (Dianova, Hamburg, Germany; 1:1000); monoclonal antibody (mAb) against CDV nucleoprotein (CDV-NP; clone 3991; 1:6000; [11]); and anti-CD44 mAb [3]. Prior to incubation with the primary antibodies paraffin sections were rehydrated through graded alcohols. Endogenous peroxidase was blocked with 0.5% H2O2 diluted in TRIS-buffered saline for 30 min at RT. Sections were incubated successively with the primary antibodies overnight at 4°C followed by the secondary antibodies [biotinylated horse-anti-mouse (CDV; Vector Labs, Burlingame, Calif.; BA2000); biotinylated rabbit-anti-rat (CD44; Vector Lab; BA4000), biotinylated goat-anti-rabbit (GFAP and MBP; Vector Lab; BA1000)] and avidin-biotin-peroxidase complex (ABC; Vector Labs) for 30 min at RT. Following incubation with the chromogen 3,3′-diaminobenzidine-tetrahydrochloride (DAB)-H2O2 in 0.1 M imidazole, pH 7.1, sections were slightly counterstained with hematoxylin. Specificity of the signals was ensured by omission of the primary and secondary antibodies or the ABC. Negative controls included replacement of the primary antibody by ascites from non-immunized BALB/c mice, rabbit normal serum, and rat serum.

Evaluation was performed semiquantitatively and included up- and down-regulation (mild, moderate, severe) compared to control tissue sections.

**Immunoelectron microscopy**

Tissue was dehydrated through graded alcohols followed by incubation in a gradually increasing concentration of LR Gold (Polysciences, no. 17412, Warrington, PA.) at −25°C. Incapsulation was performed in 100% LR Gold after addition of an initiator (Benzoin methyl ether, Polysciences, no. 00425) overnight at −25°C and for 25 h under UV light at −25°C. Sections, 90 nm thick, were cut and placed on nickel grids (PLANO, Marburg, Germany; G2200 N; 200 mesh; 3.05 mm). After a 25-min incubation with 0.5% gelatin and 1% BSA in PBS followed by a washing step, and a 20-min blocking step with goat serum, grids were placed on top of a 50-μl drop of the primary antibody dilution (anti-CD44 mAb; 1:200) and kept overnight at 4°C. As secondary antibody, mouse-anti-rat IgG (Dianova; no. 212-005-102; 1:100) was used followed by a goat-anti-mouse antibody conjugated with 5-nm gold particles (PLANO; no. 9644; 1:40). Sections were washed and incubated with anti-GFAP pAb (1:500) for 1 h. The secondary antibody was a goat-anti-rabbit antibody coupled with 18-nm gold particles (Dianova; no. 111-215-144). Sections were contrasted with saturated uranyl acetate and dried. Evaluation was performed with an EM-10C (Carl Zeiss, Oberkochen, Germany) electron microscope.

### Results

**Immunohistology**

In controls, CD44 was diffusely distributed in the white matter. The expression was most prominent periventricularly, in the medullary vela and subpially. Ependymal cells were negative. Furthermore, endothelial cells, single leptomeningeal cells and fragments of the membrana limitans gliae-forming cell processes in the molecular layer of the cerebellum were rarely positive.

In acute distemper lesions, a slight gliosis and vacuolation were the only visible lesions in H&E-stained sections. CDV antigen (CDV-NP) was mainly expressed on astrocytes. Demyelination was absent. CD44 was slightly up-regulated and there was a mildly increased GFAP expression on astrocytic cell bodies and processes.

Subacute demyelinating lesions showed pallor and a marked gliosis in H&E stained sections. CDV-NP expression was most prominent on astrocytes evenly distributed within the lesion (Fig. 1A). The plaque was almost completely demyelinated (Fig. 1B). CD44 was strongly up-regulated in the corresponding area, changing from the fine granular homogeneous distribution in controls to a thick, cord-like expression pattern (Fig. 1C). GFAP was slightly to moderately up-regulated on astrocytic cell bodies and processes within and in the periphery of the lesion (Fig. 1D).