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Up-regulation of the hyaluronate receptor CD44 in canine distemper demyelinated plaques

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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

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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 immunohistology, 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, Glasbearbeitungswerk, 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; [1]); 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% H_2O_2 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)-H_2O_2 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/cJ 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. 1 A). The plaque was almost completely demyelinated (Fig. 1 B). 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. 1 C). GFAP was slightly to moderately up-regulated on astrocytic cell bodies and processes within and in the periphery of the lesion (Fig. 1 D).

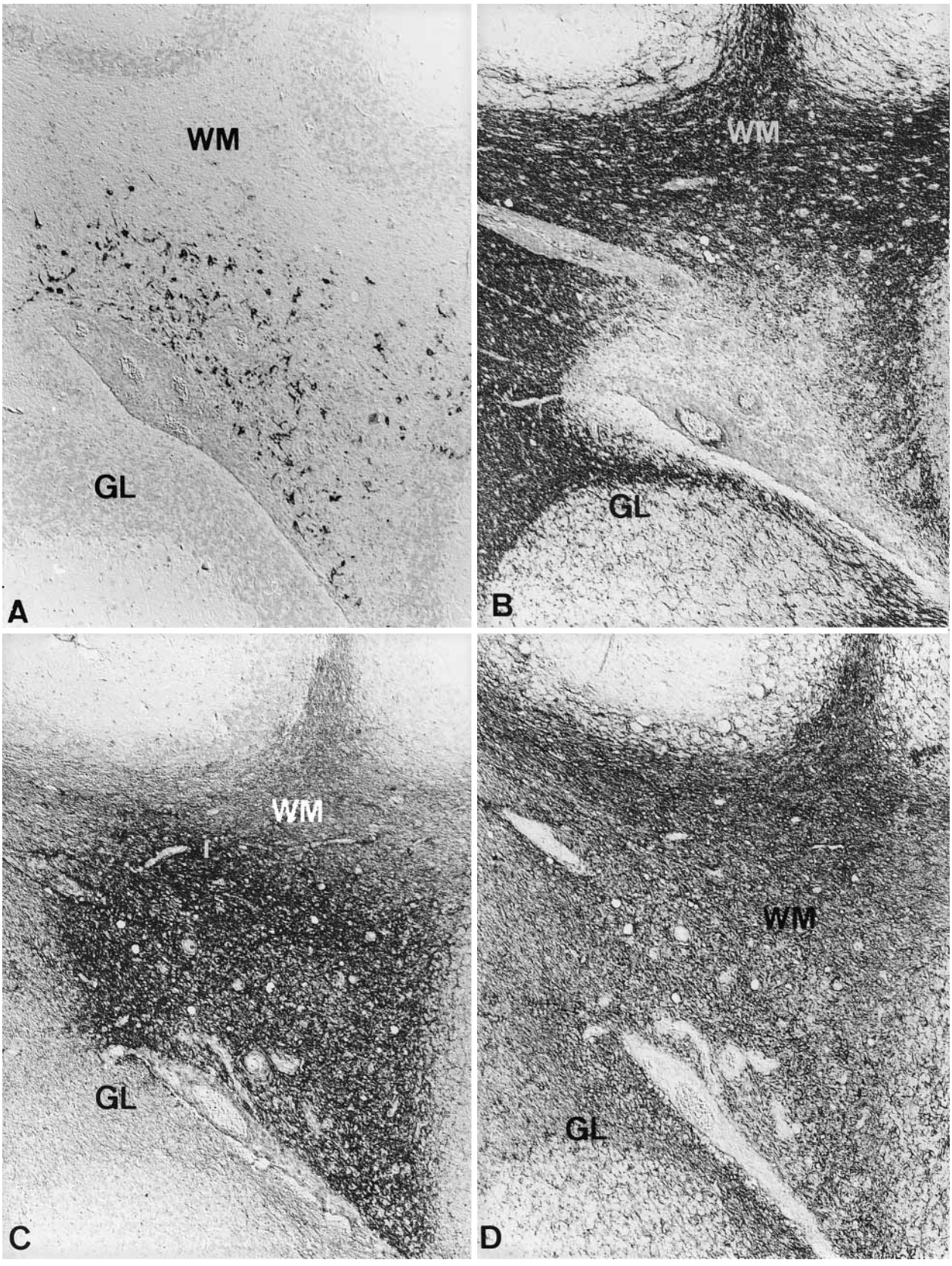

Fig. 1 A–D Cerebellum: subacute lesion. **A** CDV-NP IHC: strong expression of CDV-NP in astrocytes evenly distributed within the lesion. **B** MBP IHC: loss of myelin in the corresponding area. **C** CD44 IHC: note strong up-regulation of CD44 within the lesion corresponding to the lack of MBP staining. **D** GFAP IHC: GFAP is moderately up-regulated within and in the periphery of the lesion (*CDV-NP* Canine distemper virus nucleoprotein, *IHC* immunohistochemistry, *MBP* myelin basic protein, *GFAP* glial fibrillary acidic protein, *WM* white matter, *GL* granular layer). Serial sections, ABC method; **A–D** × 140

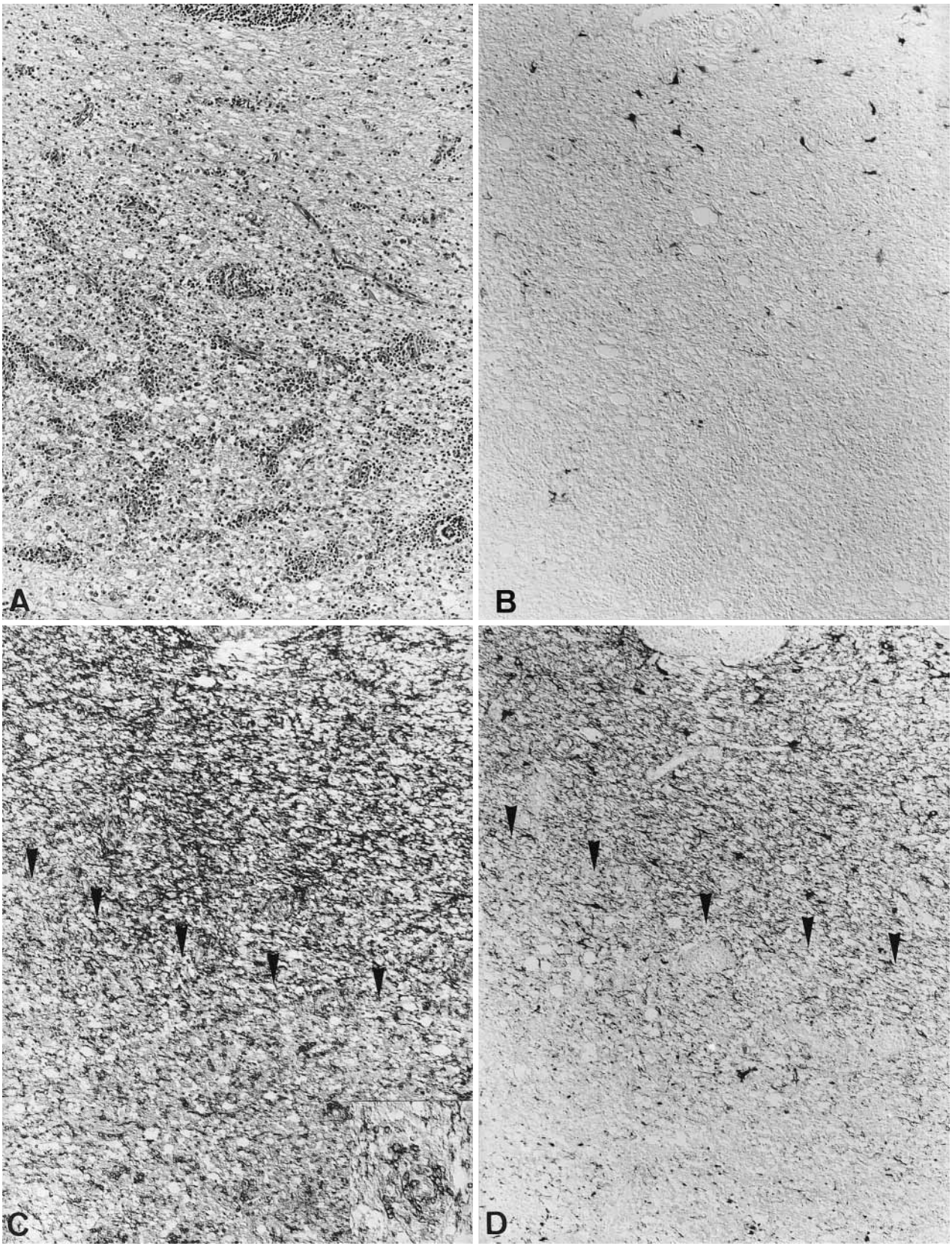

Fig. 2 A–D Cerebellum: chronic lesion. **A** H&E staining: demyelination, perivascular mononuclear infiltration, and severe gliosis. **B** CDV-NP IHC: CDV-NP expression is restricted to the periphery of the plaque. **C** CD44 IHC: reduction and loss of CD44-positive structures in the lesion; note single positive signals in perivascular mononuclear infiltrates (*insert, bottom right*). **D** GFAP IHC: note severe reduction and loss of GFAP-positive astrocytes in the lesion; *arrowheads* mark the center of the lesion. Serial sections; **B–D** ABC method; **A–D** × 140

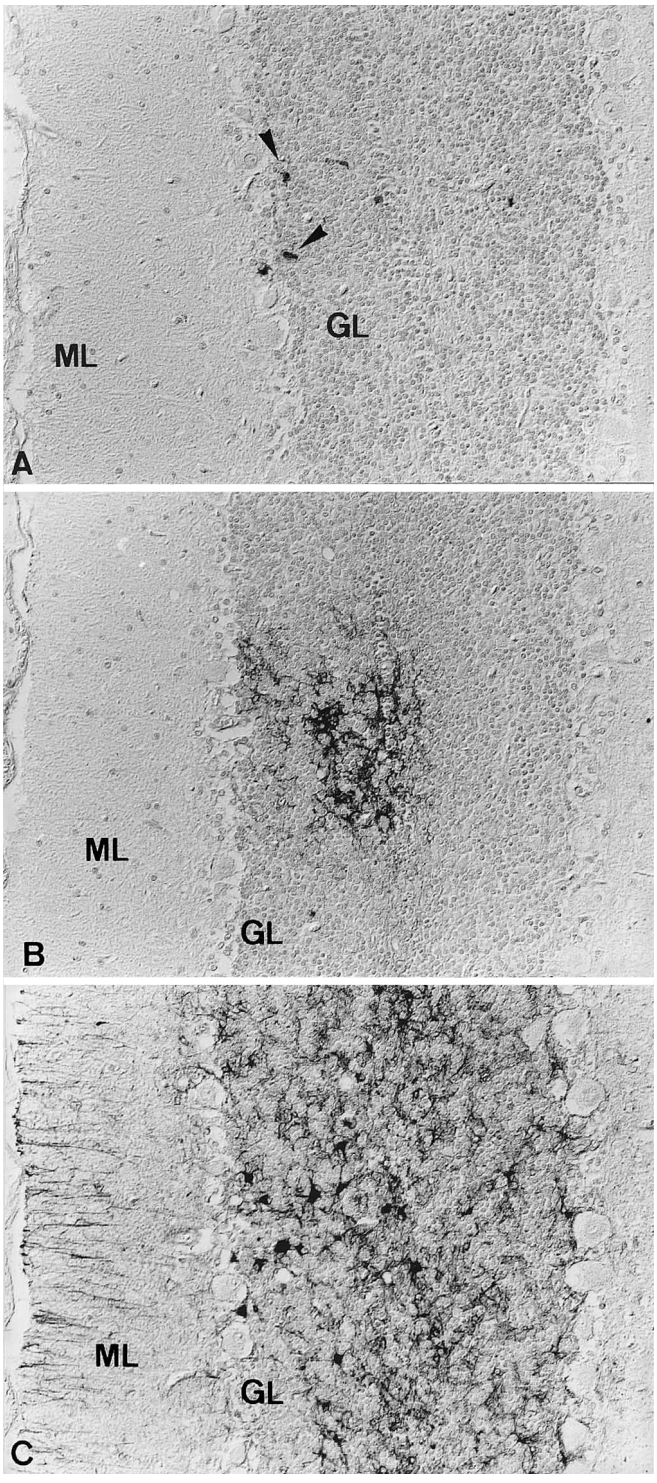

Fig. 3 A–C Cerebellum: acute lesion. **A** CDV-NP IHC: CDV expression in single cells (*arrowheads*) in the granular layer. **B** CD44 IHC: CD44 expression in the corresponding area. Note the distinct up-regulation of the receptor. **C** GFAP IHC: increased GFAP expression on single astrocytes (*ML* molecular layer, *GL* granular layer). Serial sections, ABC method; × 215

Chronic demyelinating lesions were characterized by prominent perivascular mononuclear cuffs (Fig. 2 A). CDV antigen was absent or restricted to the periphery of the lesions (Fig. 2 B). In the center of the demyelinated plaque, CD44-specific immunoreactivity was restricted to the cell membranes of mononuclear cells located perivascularly and in the neuropil and strongly reduced or absent on white matter structures (Fig. 2 C). Simultaneously, a loss of GFAP-positive astrocytes was observed (Fig. 2 D).

In addition to the white matter-associated occurrence of CD44, the receptor was found in gray matter lesions located in the granular layer of the cerebellar cortex. In H&E-stained sections only single vacuoles were detectable. CDV antigen was found in individual cells (Fig. 3 A). There were a striking up-regulation of CD44 in the corresponding area (Fig. 3 B) and an increase in the GFAP expression in the cell body and processes of single astrocytes (Fig. 3 C).

Besides the occurrence and expression pattern of CD44 in the three plaque types, the CD44-expressing cell populations were of interest. In the neuropil, CD44 was located periaxonally, and light microscopically, it was difficult to differentiate whether the receptor was expressed by myelin sheaths or by adjacent structures like astrocytic processes. In some cases, the CD44 expression could be unequivocally associated with the cytoplasm and cell membrane of the astrocytic cell body and processes (Fig. 4 A). Additionally, a focal CD44 expression on processes of Bergmann glia in single brains was noted. Small ramified cells, suggestive of microglia were also CD44 positive (Fig. 4 B) and in areas of malacia, gitter cells expressed the receptor on their cell surface (Fig. 4 C). Another CD44 expressing cell population was constituted by mononuclear inflammatory cells. In chronic lesions with prominent perivascular cuffs and active demyelination, a strong, marginal CD44 expression was found on invading inflammatory cells composed of lymphocytes, plasma cells, macrophages, and single mostly intravascularly located neutrophilic granulocytes (Fig. 4 D). Furthermore, CD44 was expressed by endothelial cells (Fig. 4 E). Ependymal cells, choroid plexus epithelial cells, and oligodendrocytes were CD44 negative.

Immunoelectron microscopy

Immunoelectron microscopy was used to specify the white matter binding sites of CD44. In controls, CD44 was rarely or not present on GFAP-expressing astrocytes and their processes. The intermediate filaments were arranged in tight bundles resulting in a high frequency of detectable GFAP molecules (Figs. 5 A, 6 A). In CDE, the astrocytic processes were broadened, leading to a lower concentration of GFAP molecules and there was a prominent up-regulation of CD44 on their cell membranes (Figs. 5 B, 6 B). There was no colocalization of GFAP and CD44, as CD44 was restricted to the cell membrane of the astrocytes, while GFAP was evenly distributed throughout the cell branches. The gold particles visualizing the localization of the hyaluronate receptor were often clustered in groups of four and more. Furthermore, it became evident that CD44 was not associated with the myelin sheath. In addition, the immunohistochemically visible expression

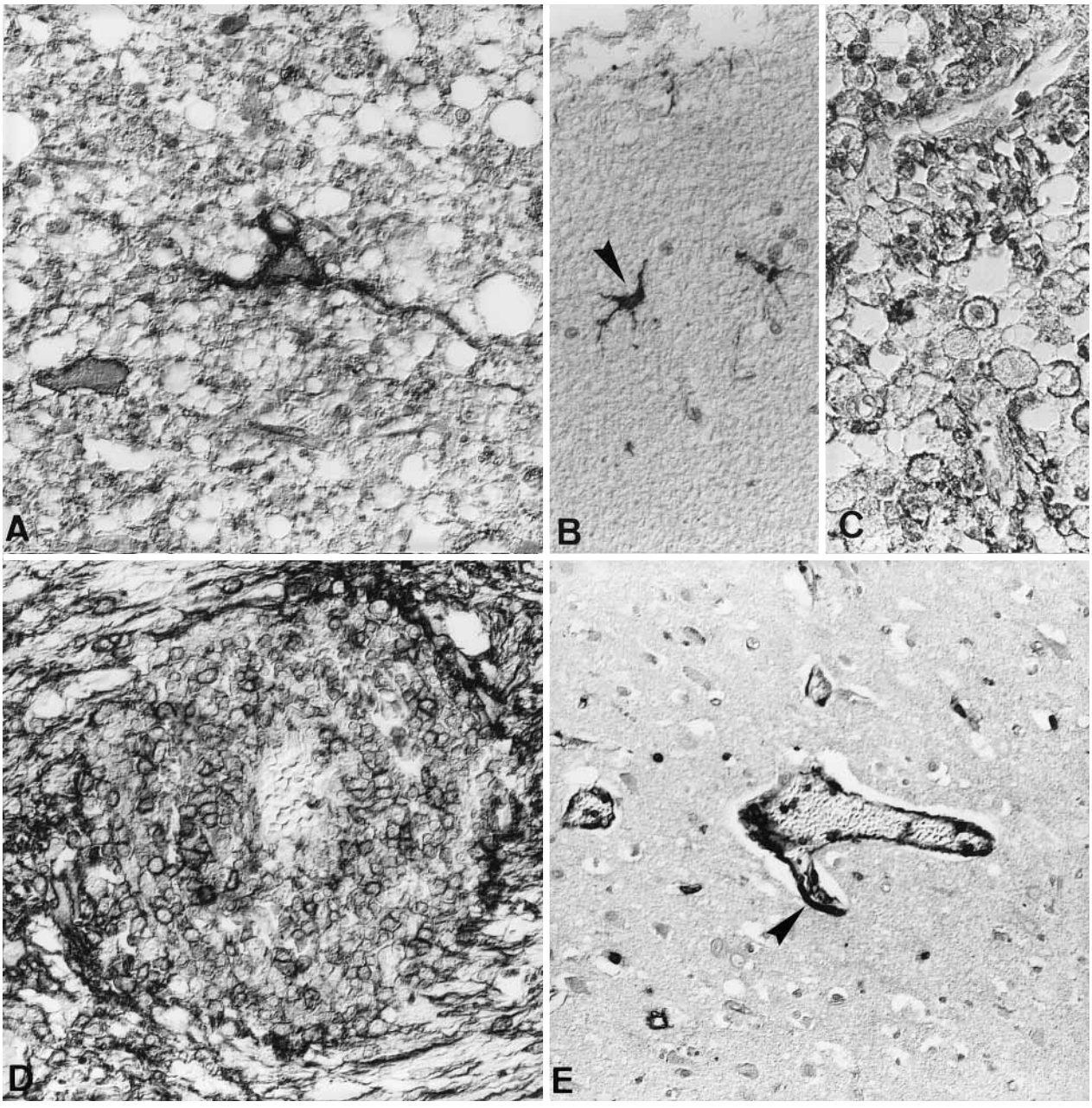

Fig. 4 **A** Cerebellum: subacute lesion. Strong marginal and cytoplasmic CD44-specific signal in white matter astrocytes. **B** Cerebellum: acute CDE: CD44 expression in cytoplasm and cell processes of cells with microglial morphology (*arrowhead*). **C** Cerebellum: subacute CDE: marginal CD44 expression on gitter cells. **D** Cerebellum: chronic CDE: numerous lymphocytes and macrophages with marginal CD44 expression. **E** Cerebrum: chronic CDE. Marked CD44 expression on endothelial cells (*arrowhead*) (*CDE* canine distemper encephalitis). **A–E** ABC method; **A–D** × 560, **E** × 280

of CD44 on cell membranes of mononuclear cells was confirmed immunoelectron microscopically.

Discussion

This study demonstrates that the receptor for hyaluronate, CD44, is up-regulated in acute and more prominent in subacute demyelinating CDE. This is in accordance with findings in brains of patients with multiple sclerosis (MS) [10]. In MS patients, CD44 was predominantly present on subependymally and subpially located fibrous astrocytes. The highest concentration was found within plaques, followed by perilesional white matter, normal white matter of an MS patient and white matter of a control brain. Reactive astrocytes in active plaques display a strong CD44 expression on their cell membranes. Cruz et al. [6] used the antibody to evaluate the concentration of the corresponding antigen in brain homogenates of MS patients and patients with other CNS diseases and found an increase of the antigen in the gray and white matter exclusively in MS patients.

In the acute and subacute phase of CDE, the increased CD44 expression is associated with a prominent GFAP immunoreactivity. As described and summarized by Gaedke

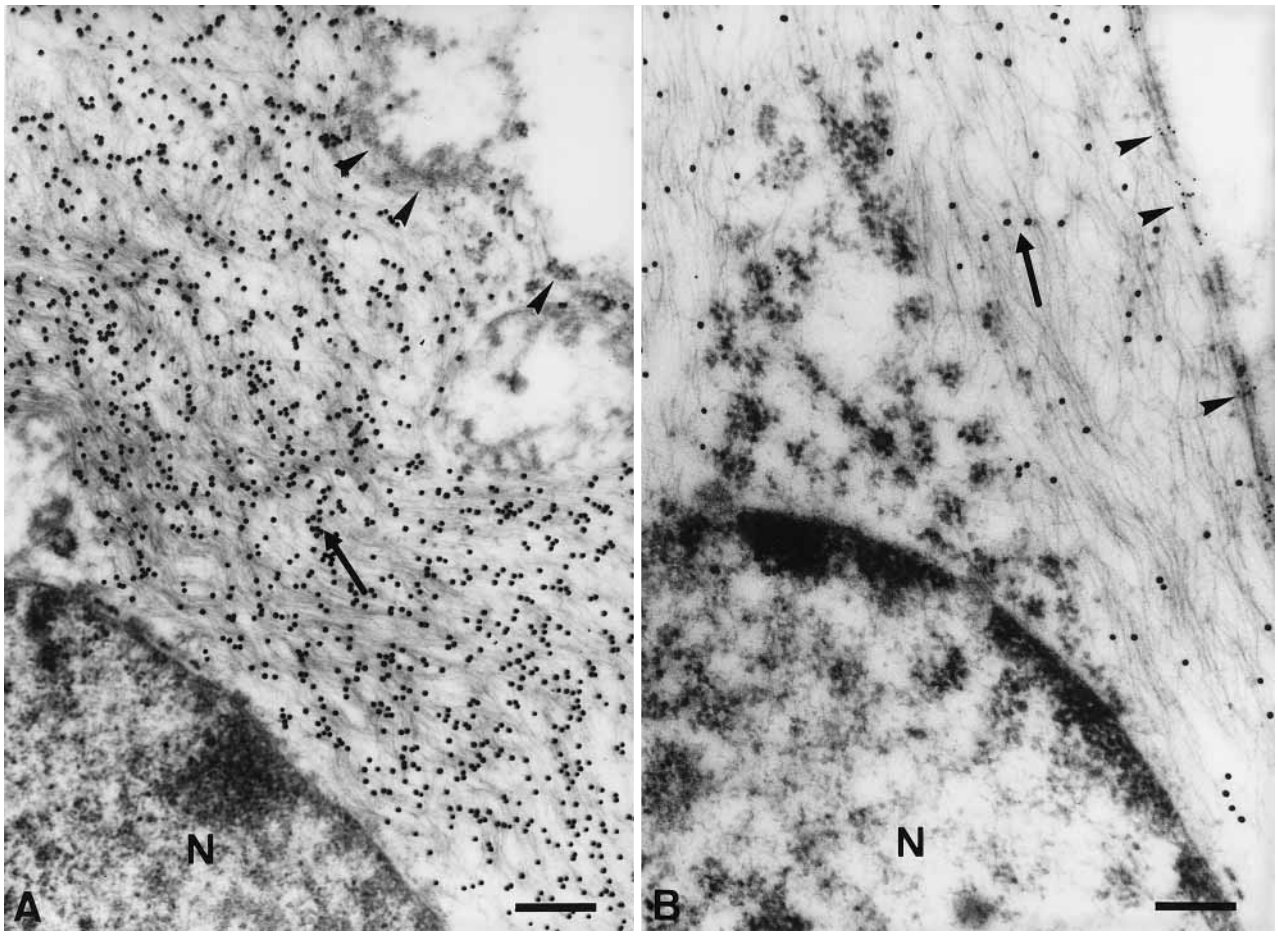

Fig. 5 A, B Cerebellum. **A** Control: section of an astrocyte: GFAP-specific signal on tightly packed cytoplasmic intermediate filaments (*arrow*). No binding of CD44-specific antibodies is found (*arrowheads*). **B** CDE: loosely arranged intermediate filaments with less frequent GFAP-specific signals (*arrow*). Prominent CD44-specific signals on the cell membrane (*arrowheads*) (*N* nucleus). **A**, **B** Immunoelectron microscopy; *bars* 360 nm

et al. [9], there is an up-regulation of GFAP on astrocytes in acute and subacute distemper lesions, while in chronic plaques, GFAP-positive astrocytes are reduced or absent and restricted to the edge of the plaque. In the present study, loss of astrocytes in chronic lesions was accompanied by a reduction of the CD44 expression, leading to the conclusion that the hyaluronate receptor must be associated with astrocytes. This assumption was verified immunoelectron microscopically by the demonstration of CD44 antigen expressed on astrocytes and their processes. In CDV infection, astrocytes are the cell population mainly affected [17]. An up-regulation of CD44 might be directly virus induced or, as suspected by Girgah et al. [10], caused by a proliferation and activation of this cell population. Cells from an astrocytoma cell line exhibited an up-regulation of CD44 after binding of MBP [19]. Furthermore, this binding with MBP was saturated by a ten-fold lower concentration compared to the hyaluronate ligation and signals astrocyte migration. Again, a prerequisite for this process is myelin damage followed by ligation and up-regulation of the CD44 receptor and a migration of astrocytes to the demyelinated lesion. Cruz et al. [5] characterized the anti-CD44 antibody used in the MS study mentioned above and showed that it reacted with loosely structured myelin and myelin fragments, but not with compact multilamellar myelin. The results of the immunoelectron microscopic investigation in this study support the observation that compact myelin is CD44 negative.

In chronic demyelinating CDE, intralesional CD44 expression was reduced or restricted to perivascularly and intralesionally located mononuclear cells. Besides lymphocytes and plasma cells, microglia of different activation states including gitter cells were CD44 positive. Girgrah et al. [10] showed that in the CNS of MS patients, blood-born macrophages are CD44 negative. According to Cruz et al. [6], microglia do not normally express the antigen, but are CD44 positive in MS brains. What are the consequences of CD44 expression of phagocytes on the demyelination process? Noble et al. [18] observed that macrophages incubated with hyaluronic acid in vitro are activated, proliferate and express cytokines. If 8-day-old bone marrow macrophages of the rat are ligated with hyaluronic acid, they show a fast production of tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β mRNA and insulin-like growth factor-1 protein synthesis [14]. Interestingly, in CDE, IL-1β was observed in acute, subacute, and chronic lesions, while TNF-α seemed to be sig-

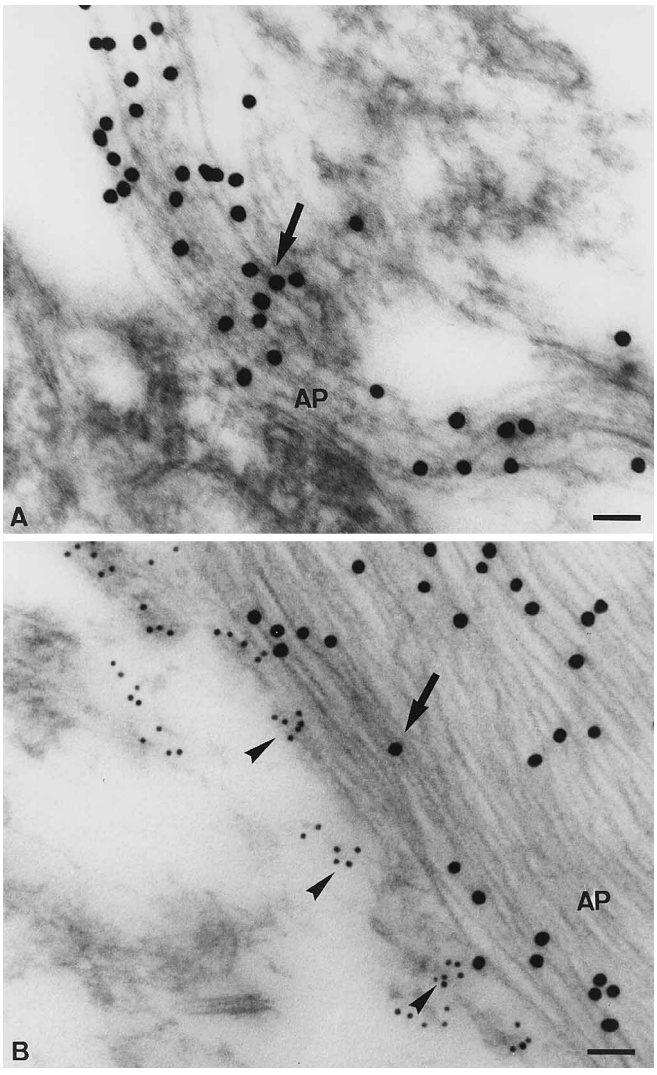

Fig. 6 A, B Cerebellum. **A** Control: GFAP-specific signal on fine, filamentous astrocytic processes. No binding of CD44-specific antibodies is found. **B** CDE: abundant expression of CD44 (5-nm gold particles; *arrowheads*) on broadened astrocytic GFAP-positive (18-nm gold particles; *arrows*) processes (*AP* astrocytic process). **A**, **B** Immunoelectron microscopy; *bars* 75 nm

nificant in early lesions with no or little inflammation (Gröne et al., personal communication). Furthermore, CD44 expression is important for lymphocyte targeting, as CD44-hyaluronic acid interactions may lead lymphocytes to specific extralymphoidal effector locations. Hence, the up-regulation of CD44 in acute and subacute CDE might enhance the inflammatory response most prominent in subacute and chronic lesions [28]. Finally, in CDE, CD44 expression may also be present on effector or memory lymphocytes [8].

In summary, this study clearly demonstrated that the hyaluronate receptor CD44 is up-regulated in acute and subacute demyelinating CDE. Following CDV induced myelin damage, CD44 plays a dual role for the progression of the demyelination process. Ligation of the receptor on macrophages/microglia might induce chemokines and cytokines, and hence initiate and perpetuate the inflammatory process. Secondly, the binding of MBP on the CD44 receptor on fibrous astrocytes might up-regulate CD44 and allow the migration of this cell population by the interaction with the ECM. CD44-positive, perivascularly located cells in CDE might be memory cells. In conclusion, CD44 is involved in the initial demyelination processes in the early phase of CDE and seems to be of importance in the late phase as activity marker of invading immune cells.

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