

# A new paraclinical CSF marker for hypoxia-like tissue damage in multiple sclerosis lesions

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## Summary

Recent studies on the immunopathology of multiple sclerosis revealed a heterogeneity in the patterns of demyelination, suggesting interindividual differences in the mechanism responsible for myelin destruction. One of these patterns of demyelination, characterized by oligodendrocyte dystrophy and apoptosis, closely mimics myelin destruction in acute white matter ischaemia. In the course of a systematic screening for virus antigen expression in multiple sclerosis brains, we identified a monoclonal antibody against canine distemper virus, which detects a cross-reactive endogenous brain epitope, highly expressed in this specific subtype of actively demyelinating multiple sclerosis lesions with little or no

immunoreactivity in other active multiple sclerosis cases. The respective epitope, which is a phosphorylation-dependent sequence of one or more proteins of 50, 70 and 115 kDa, is also expressed in a subset of active lesions of different virus-induced inflammatory brain diseases, but is present most prominently and consistently in acute lesions of white matter ischaemia. Its presence is significantly associated with nuclear expression of hypoxia-inducible factor-1 $\alpha$  within the lesions of both inflammatory and ischaemic brain diseases. The respective epitope is liberated into the CSF and, thus, may become a useful diagnostic tool to identify clinically a defined multiple sclerosis subtype.

**Keywords:** multiple sclerosis; demyelination; hypoxia; cerebrospinal fluid; canine distemper

**Abbreviations:** BND = bacterial-induced neurological disease; CDV = canine distemper virus; ELISA = enzyme-linked immunosorbent assay; FBS–PBS = fetal calf serum–phosphate-buffered saline; HIF-1 $\alpha$  = hypoxia-inducible factor-1 $\alpha$ ; Ig = immunoglobulin; OND: non-inflammatory neurological diseases; PBS = phosphate-buffered saline; PBS-T = phosphate-buffered saline–0.05% Tween-20; PCR = polymerase chain reaction; PLP = proteolipid protein; SDS = sodium dodecylsulfate; VND = viral-induced neurological disease

## Introduction

By analysing a large sample of biopsy and autopsy cases of multiple sclerosis in the active stage of demyelination, we were able to define four distinctly different patterns of demyelination, which were heterogenous between patients, but homogenous within multiple active lesions of a single patient (Lucchinetti *et al.*, 1999, 2000). This observation may have therapeutic consequences, when it is possible to identify these different pathways of demyelination by clinical or paraclinical markers in the living patient.

One specific pattern of demyelination is defined by an oligodendroglialopathy, primarily affecting the most distant processes of these cells and leading to apoptotic cell death of oligodendrocytes at later stages of lesion development (Lucchinetti *et al.*, 2000). These alterations of oligodendrocytes and myelin are similar to those present in acute ischaemic white matter damage and are associated with nuclear expression of hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ). Here we describe that a monoclonal antibody against the

**Table 1** Clinical data

| Tissue samples      | <i>n</i> | M/F ratio | Age (years)* | Disease duration*           | Cells*      | IgG Ind*  | Q-Alb*  |
|---------------------|----------|-----------|--------------|-----------------------------|-------------|-----------|---------|
| Acute MS            | 11       | 1.2       | 48 ± 11      | 6.5 ± 6.7 weeks             |             |           |         |
| Chronic active MS   | 12       | 3.5       | 39 ± 11      | 4.5 ± 3 years               |             |           |         |
| Chronic inactive MS | 11       | 2         | 68 ± 11      | 25.9 ± 8.2 years            |             |           |         |
| CMV enceph.         | 8        | 0 (8 M)   | 40 ± 10      | 11.3 ± 3.5 weeks            |             |           |         |
| HSV enceph.         | 6        | 0.5       | 48 ± 24      | 2.6 ± 0.9 weeks             |             |           |         |
| PML                 | 11       | 0.1       | 41 ± 9       | 31.2 ± 30.3 weeks           |             |           |         |
| FSME                | 5        | 0.6       | 61 ± 12      | 3.9 ± 3.2 weeks             |             |           |         |
| HIV enceph.         | 5        | 0 (5 M)   | 38 ± 7       | 13.5 ± 3.5 weeks            |             |           |         |
| SSPE                | 2        | 1         | 30 ± 12      | 40; >500 weeks              |             |           |         |
| Stroke              | 13       | 1.2       | 80 ± 11      | 29.8 ± 53.2 weeks           |             |           |         |
| Controls            | 9        | 0.8       | 69 ± 22      | NA                          |             |           |         |
| Myo. infarcts       | 8        | 1.67      | 54 ± 7       | 41 ± 28 months <sup>+</sup> |             |           |         |
| CSF studies         |          |           |              |                             |             |           |         |
| MS R0               | 43       | 1.6       | 33 ± 11      | 0 ± 0 years                 | 9 ± 25      | 1.1 ± 0.6 | 5 ± 3   |
| MS RR               | 25       | 3.1       | 34 ± 8       | 4 ± 3 years                 | 5 ± 5       | 1.1 ± 0.9 | 5 ± 1   |
| MS SP               | 14       | 2.5       | 47 ± 13      | 17 ± 11 years               | 3 ± 8       | 1.1 ± 0.9 | 8 ± 7   |
| MS PP               | 10       | 1         | 51 ± 12      | 6 ± 5 years                 | 5 ± 5       | 0.9 ± 0.5 | 7 ± 2   |
| Stroke              | 12       | 1         | 58 ± 15      | 8 ± 6 days                  | 9 ± 25      | 0.6 ± 0.4 | 18 ± 43 |
| VND                 | 13       | 1.14      | 52 ± 17      | 12 ± 13 days                | 80 ± 124    | 0.6 ± 0.3 | 10 ± 9  |
| BND                 | 10       | 9         | 47 ± 23      | 5 ± 3 days                  | 2028 ± 2723 | 0.7 ± 0.2 | 17 ± 32 |
| OND                 | 46       | 1.42      | 48 ± 15      | 5 ± 4                       | 1 ± 1       | 0.5 ± 0.1 | 2 ± 2   |
| BD                  | 31       | 1.07      | 45 ± 10      |                             |             |           |         |

Cells = CSF cells/μl; IgG Ind = CSF IgG index; Q-Alb = albumin quotient; R0 = first attack; RR = relapsing–remitting disease course; SP = secondary chronic progressive disease course; PP = primary chronic progressive disease course; VND = viral neurological diseases; BND = bacterial neurological diseases; OND = other neurological diseases; BD = blood donors; Myo. infarcts = myocardial infarcts; MS = multiple sclerosis; CMV encephal. = cytomegalovirus encephalitis; HSV encephal. = herpes simplex virus encephalitis; FSME = European tick-borne encephalitis; HIV encephal. = human immunodeficiency virus encephalitis; PML = progressive multifocal leucoencephalopathy; NA = not available. \*Mean values with SDs. <sup>+</sup>Clinical duration of heart disease; the samples contained fresh myocardial infarcts at different stages of lesion development.

nucleocapsid protein of canine distemper virus (CDV) cross-reacts with an antigen which is expressed with a high degree of selectivity in these particular multiple sclerosis patients as well as in acute stroke lesions. Since the antigen is liberated into the CSF, the antibody may become a useful paraclinical tool to identify a pathogenetically distinct subtype of multiple sclerosis patients.

## Material and methods

### Autopsy and biopsy cases

The study was performed on archival paraformaldehyde-fixed paraffin-embedded tissue samples from 101 patients, collected at the Brain Research Institute of Vienna University. Clinical diagnosis and demographic data are summarized in Table 1.

The multiple sclerosis population contained all different clinical variants of the disease. Furthermore, all different patterns of demyelination, as defined by Lucchinetti *et al.* (2000), were included in the sample. Two to five representative tissue blocks were investigated per case. Active demyelination within the lesions was classified according to Brück *et al.* (1995) by the presence of macrophages with myelin degradation products, immunoreactive for myelin proteins.

In addition to these autopsy cases, four brain biopsies, performed for diagnostic reasons, were available (Table 2). In these patients, multiple serum and CSF samples were collected at various time points of disease development. All biopsy patients suffered from an acute, fulminant neurological disease with multifocal large lesions in the cerebral white matter. Biopsy diagnosis revealed an acute inflammatory demyelinating process, consistent with acute multiple sclerosis. More detailed immunopathological classification showed that three patients had lesions compatible with antibody-mediated demyelination, while the other one contained lesions with distal oligodendroglialopathy and oligodendrocyte apoptosis.

### CSF samples

CSF samples were obtained for diagnostic purposes between 1993 and 2001 from patients who gave informed consent, and were stored at -20°C until use. The clinical and CSF data of all patients analysed in this study are shown in Table 1. Multiple sclerosis patients (*n* = 93) were diagnosed according to the criteria of Poser *et al.* (1983). In addition, 12 patients with cerebrovascular disease (stroke), 13 patients with viral neurological diseases (VND; viral meningitis, meningoencephalitis and encephalitis), 10 patients with bacterial neurological diseases (BND; bacterial meningitis and

**Table 2** Expression of D-110 epitope in the CSF of acute multiple sclerosis patients

| Patient/age/gender         | Disease duration before biopsy  | Date of biopsy | Date of CSF examination | Biopsy pathology | CSF D-110 (OD units) |
|----------------------------|---|----------------|-------------------------|------------------|----------------------|
| B/22/female active disease | 4 months  | 2/17/00        | 8/17/00                 | MS pattern II    | 0.233                |
| K/26/male active disease   | 1 month   | 3/3/99         | 2/26/99                 | MS pattern II    | 0.244                |
| Ha1/24/male                | MRI after car accident revealed multiple enhancing supratentorial lesions | 4/12/01        | 3/29/01                 | MS pattern II    | 0.248                |
| Ha2                        |   |                | 6/13/01                 |                  | 0.217                |
| Ha3                        |   |                | 7/2/01                  |                  | 0.232                |
| Ha4                        |   |                | 10/5/01                 |                  | 0.248                |
| Kr1/33/male active disease | 1 month   | 5/4/00         | 5/9/00                  | MS pattern III   | 0.419                |
| Kr2 active disease         |   |                | 5/16/00                 |                  | 0.422                |
| Kr3 remission              |   |                | 5/29/00                 |                  | 0.250                |

MS pattern II = multiple sclerosis pattern II demyelination; MS pattern III = multiple sclerosis pattern III demyelination. CSF samples Ha2, 3, 4, Kr2 and 3 are follow up samples of patients Ha1 and Kr1, respectively.

**Table 3** CDV antibodies for immunohistochemistry

| Monoclonal antibody | CDV protein | Reference                       |
|---------------------|-------------|---------------------------------|
| D-110               | NP          | Hamburger <i>et al.</i> (1991)  |
| D-15                | NP          | Dubacher (1988)                 |
| D-150               | NP          | Dubacher (1988)                 |
| a3H-10              | NP          | Hamburger <i>et al.</i> (1991)  |
| K4-2                | NP          | Dubacher (1988)                 |
| 3991                | NP          | Dubacher (1988)                 |
| 4996                | NP          | Dubacher (1988)                 |
| 3570                | NP          | Dubacher (1988)                 |
| XI-6                | M           | Dubacher (1988)                 |
| XI-23               | H           | Dubacher (1988)                 |
| CDV probes for ISH  | mRNA        | Reference                       |
| NP-3                | NP          | Zurbriggen <i>et al.</i> (1995) |
| NP-1                | NP          | Zurbriggen <i>et al.</i> (1995) |
| P-3                 | P           | Müller <i>et al.</i> (1995)     |

ISH = *in situ* hybridization; NP = nucleocapsid protein N epitope; M = nucleocapsid protein M; H = glycoprotein H; P = P-specific sequence of CDV.

meningoencephalitis) and 46 patients with other neurological diseases (OND; headache, back pain and disc protrusion) were included in this study.

## Antibodies and reagents

### CDV antibodies

Monoclonal antibody D-110 detects an epitope within the C-terminus (between amino acids 412 and 523) of the CDV nucleocapsid (N) protein. This antibody binds to all CDV strains (Bollo *et al.*, 1986; Hamburger *et al.*, 1991). Six additional monoclonal antibodies recognizing other epitopes of the N protein, two monoclonal antibodies binding to the

surface glycoprotein H and one monoclonal antibody binding to the auxiliary nucleocapsid protein M were included (Table 3). The specificity of all antibodies was determined by immunoprecipitation and on western immunoblots, and their suitability for the detection of CDV in paraffin sections was established in dog brain lesions of CDV encephalitis (Müller *et al.*, 1995; Zurbriggen *et al.*, 1995). Since D-110 is a mouse immunoglobulin Ig G1 antibody, we used other IgG1 antibodies against irrelevant antigens as controls for immunocytochemistry and western blotting.

### CDV *in situ* hybridization probes

For *in situ* hybridization, digoxigenin-labelled RNA probes were used (Table 3; Zurbriggen *et al.*, 1995). Two probes complementary to the N-specific sequence of the virulent A75/17 CDV were selected, because the D-110 antibody recognizes an epitope within the N protein of CDV. A probe complementary to the P-specific sequence of CDV (P in Table 3) was chosen, because it has been shown to be the most sensitive marker for CDV RNA in distemper. All probes have been tested positive on paraformaldehyde-fixed paraffin-embedded tissue samples from dogs with canine distemper encephalitis.

### Other immunocytochemical markers

Primary antibodies were used against the following targets: myelin oligodendrocyte glycoprotein (Piddlesden *et al.*, 1993), myelin-associated glycoprotein (D7E10 and B11F7; Doberson *et al.*, 1985), cyclic nucleotide phosphodiesterase (Affinity Research Products, Manhead Castle, Exeter, UK), proteolipid protein (PLP; Serotec, Oxford, UK) and HIF-1 $\alpha$  (BD Transduction Laboratories, Lexington, KY, USA).

### Neuropathology and immunocytochemistry

Paraffin sections were stained with haematoxylin/eosin, luxol fast blue for myelin and Bielschowsky silver impregnation for axons. Immunocytochemistry was performed with a biotin-avidin technique (Vass *et al.*, 1986). Microwave pre-treatment was used in tissue samples with prolonged formaldehyde fixation time. Non-radioactive *in situ* hybridization was used with digoxigenin-labelled riboprobes using the Boehringer Kit (Breitschopf *et al.*, 1992). For dephosphorylation, sections were pre-treated with alkaline phosphatase (Sigma Type VII-L, 400 µg/ml in 0.1 M Tris-HCl pH 8, containing 10 mM phenylmethylsulfonyl fluoride) for 18 h at 37°C (Bancher *et al.*, 1989).

The number of cells reactive for D-110 and HIF-1 $\alpha$  was determined in zones of active myelin destruction in 10 standardized microscopic fields of 10 000 µm<sup>2</sup> each, defined by an ocular morphometric grid.

### Identification of CDV and paramyxovirus using RT-PCR

Total RNA was isolated from five sections per case (5 µm thick) of paraffin-embedded formalin-fixed brain tissue as described elsewhere (Santa *et al.*, 1998). An aliquot of 3–5 µl of total RNA was reverse transcribed (RT) and polymerase chain reaction (PCR) amplified using a Gene Amp RNA PCR Core Kit (Perkin Elmer Biosystems). From each individual RNA extraction, we performed RT-PCR to amplify a PLP-specific, 124 bp PCR product using primers Oli.396 5' GTGGACATGAAGCTCTCACTG 3' and Oli.301 5'GCAGTCCATAGATGACATACTGGA 3', flanking PLP intron 2, as an RNA quality control. We designed paramyxovirus (measles virus)-specific PCR primers Oli.422 5' ATGGGGGAAACTGCACCTTACATGGT 3' and Oli.398 5' CCTCCCATGGAGTTTTCAAGTTC 3' in a highly conserved region of the nucleocapsid protein at nucleotides 1023–1152 of CDV, complete genome (accession number gi3335048). PLP and paramyxoviridae virus-specific PCRs were performed under standard conditions at an annealing temperature of 61 and 59°C, respectively. For the detection of paramyxovirus RNA from paraffin-embedded human tissue, we always performed a boosted PCR. In these cases, 1 µl of a 1 : 50 dilution of the first PCR was used as a template for the second PCR. The PLP-specific RT-PCR was performed as a positive control insuring RNA quality, and a boosted PCR without template was used as a negative control.

### Western blot analysis and enzyme-linked immunosorbent assay

Western blot analysis was performed using standard methods. Briefly 13 µl of CSF (~5 µg of CSF total protein) were loaded per well and separated in 10% BisTris (NuPage) sodium dodecyl sulfate (SDS)-polyacrylamide gels (Novex, San Diego, CA, USA). Separated proteins were electrotransferred

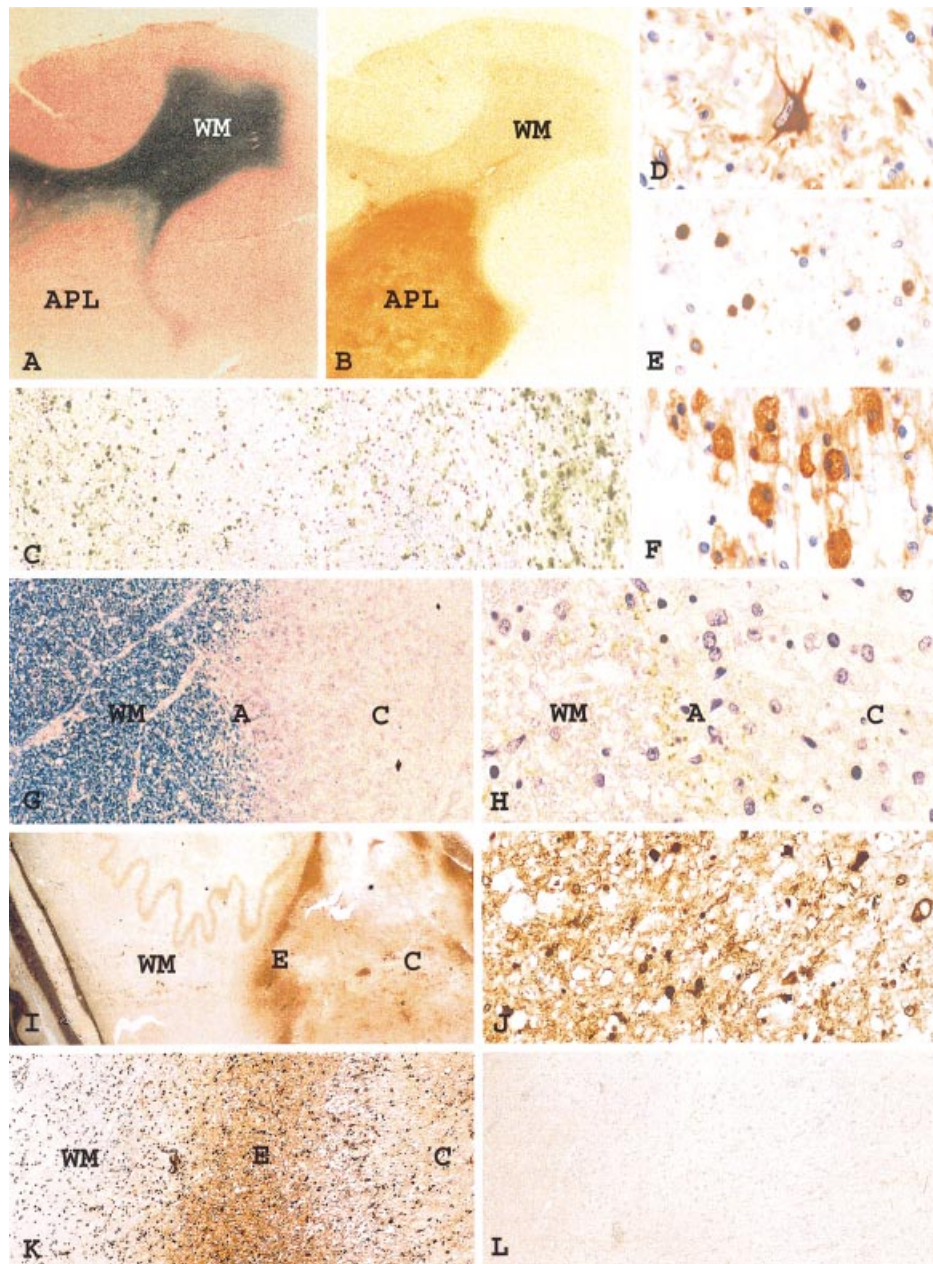
to nitrocellulose membranes (Hybond-C, Amersham Pharmacia Biotech, Uppsala, Sweden). Efficiency of transfer was monitored by the use of a pre-stained low range SDS-polyacrylamide gel electrophoresis (PAGE) standard (Bio-Rad, Hercules, USA) and by staining of the filters with Ponceau S (Sigma, St Louis, MO, USA) after transfer. Blots were blocked with 2% milk powder in phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBS-T) and probed with D-110 or HIF-1 $\alpha$  antibodies (1 : 500 in 2% milk powder in PBS-T) overnight at 4°C. Blots were washed three times with PBS-T and incubated with horseradish peroxidase-conjugated anti-mouse IgG (1 : 5000; Amersham Pharmacia Biotech) for 1 h at room temperature. After washing, bound antibodies were detected by enhanced chemoluminescence (Amersham Pharmacia Biotech).

To compare D-110 antibody reactivity to phosphorylated and dephosphorylated proteins, the blots were treated with 15 U/ml alkaline phosphatase (type III; Sigma, St Louis, MO, USA) in 100 mM Tris-HCl pH 9.5, 50 mM MgCl<sub>2</sub>, 100 mM NaCl overnight at 37°C. After three washes with PBS-T, dephosphorylated proteins were incubated with D-110 antibody as described above.

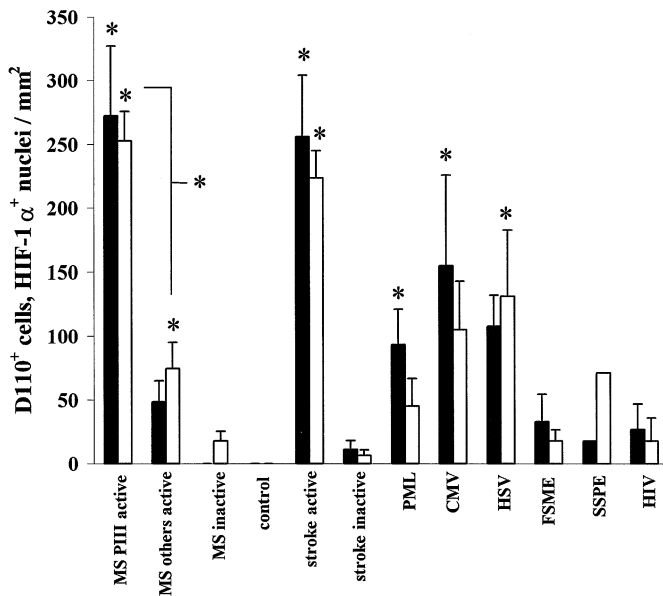
For enzyme-linked immunosorbent assay (ELISA), CSF proteins were covalently coupled to protein immobilizer plates (Exiqon, Vedbaek, Denmark). To this end, 25 µl of CSF was applied to immobilizer plates in 100 µl of 100 mM carbonate buffer pH 9.6 for 2 h at room temperature. CSF proteins were then incubated for 15 min with 200 µl of 2% SDS in PBS. After six washes with PBS-T, the plates were incubated with 100 µl of D-110 or HIF-1 $\alpha$  antibody/well (1 : 100 in PBS-T) overnight at 4°C, washed six times with PBS-T and incubated with alkaline phosphatase-coupled anti-mouse IgG (1 : 3000; Axell, Westbury, USA) for 1 h at room temperature. After washing, the reaction products were visualized with *p*-nitrophenylphosphate (Sigma) in 1 M diethanolamine, 5 mM MgCl<sub>2</sub> pH 9.8, and the optical density determined at 405 nm. Control wells were incubated with secondary antibody alone (no primary antibody) and all data were corrected by subtraction of these background values. Variations between single microtitre plates were determined using CSF samples with (known) high and low D-110 values throughout all ELISA plates used. Both the inter- and intra-assay variations were <15%.

CDV nucleocapsid protein was expressed in the pET14 expression system (Novagen, Madison, WI, USA). The recombinant CDV nucleocapsid protein contains an N-terminal histidine tag in order to allow affinity purification on an Ni-NTA-agarose column.

For production of recombinant CDV nucleocapsid protein, *Escherichia coli* strain BL21(DE3) containing the N/pET14 plasmid, containing the entire CDV nucleocapsid gene, were grown until an optical density (at 600 nm) of 0.6 was reached. Expression of the recombinant protein was then induced by the addition of 0.4 mM isopropyl- $\beta$ -D-thiogalactopyranoside (Roche Molecular Biochemicals, Mannheim, Germany) and bacteria were grown for an additional 4 h. Bacteria were



**Fig. 1** Expression of D-110 antigen in multiple sclerosis and stroke lesions. (A–F) Large acute multiple sclerosis lesion in the subcortical white matter with very intense expression of D-110 antigen. The actively demyelinating lesion was present in a 45-year-old male patient with a clinical history of 3 weeks and followed lesional pattern III according to Lucchinetti *et al.* (2000). APL = acute demyelinating plaque; WM = normal white matter. **A** shows the outline of the lesion in luxol fast blue myelin stain, while **B** documents the intense D-110 immunoreactivity (brown) within the whole plaque area (APL). Higher magnification of the section shown in **B** reveals a high density of brown immunoreactive cells (**C**), which in higher magnification can be identified as (**D**) astrocytes, (**E**) oligodendrocytes and (**F**) macrophages. Magnifications: **A** and **B**,  $\times 5$ ; **C**,  $\times 50$ ; **D**, **E** and **F**,  $\times 400$ . (**G** and **H**) A chronic active multiple sclerosis lesion with very low expression of the D-110 antigen. This lesion was present in a 28-year-old male patient with 400 week disease duration and followed pattern II (compatible with antibody-mediated demyelination) as defined by Lucchinetti *et al.* (2000). WM = periplaque white matter; A = zone of active demyelination with granular myelin degradation products in macrophages; C = inactive plaque centre. **G** shows the lesion outline in luxol fast blue myelin stain, and **H** the immunoreactivity for D-110, which reveals minimal staining in some cell processes in the zone of active demyelination (A). Magnifications: **G**,  $\times 100$ ; **H**,  $\times 800$ . (**I–L**) Acute infarct in the cerebellar white matter, stained by immunocytochemistry for D-110 antigen; there is massive expression of the antigen at the edge of the infarct (E), while the necrotic centre (C) shows much less reactivity; there is no reactivity in the perilesional white matter (WM). At higher magnification, D-110 immunoreactivity is concentrated at the edge of the infarct (**K**) and expressed in different cell types in the lesion (**J**). When the section is pre-treated with alkaline phosphatase, immunoreactivity is completely abolished (**L**). Magnifications: **I**,  $\times 2$ ; **J**,  $\times 200$ ; **K** and **L**,  $\times 30$ .



**Fig. 2** Average number of cells with D-110 (black bars) and HIF-1 $\alpha$  (white bars) immunoreactivity in the lesions of different brain diseases and normal controls. MS PIII active = actively demyelinating multiple sclerosis lesions with pattern III demyelination; MS others active = actively demyelinating multiple sclerosis lesions with other patterns of demyelination (patterns I, II and IV); MS inactive = inactive lesions from multiple sclerosis patients; control = normal brain from control patients; stroke active = early stroke lesions (1 week disease duration); stroke inactive = inactive stroke lesions (>6 weeks disease duration); HSV = herpes simplex encephalitis; PML = progressive multifocal leucoencephalopathy; CMV = cytomegalovirus encephalitis; FSME = European tick-borne encephalitis; HIV = human immunodeficiency virus encephalitis; SSPE = subacute sclerosing panencephalitis. \* $P < 0.01$  in comparison with controls as well as between MS PIII active and MS others active.

harvested by centrifugation and lysed in urea buffer (8 M urea, 100 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris-HCl) pH 8.0. Crude lysates were then cleared by centrifugation and the supernatant was applied to an Ni-NTA-agarose column (Qiagen, Hilden, Germany) under denaturing conditions in urea buffer. Bound fusion proteins were eluted by a shift in pH from 8 to 4.5 and stored at -20°C.

Anti-CDV nucleocapsid protein serum IgM and IgG antibodies were measured by ELISA using 96-well Nunc-Immuno Maxisorp microtitre plates (Nunc, Roskilde, Denmark) coated with 100  $\mu$ l of a 10  $\mu$ g/ml recombinant CDV nucleocapsid protein solution in PBS overnight at 4°C (1  $\mu$ g/well). A 100  $\mu$ l aliquot of 10% fetal bovine serum (FBS; Life Technologies) in PBS (FBS-PBS) was used as background sample. Plates were then washed six times with PBS-T and blocked with FBS-PBS for 2 h at room temperature. After washing, 100  $\mu$ l of diluted serum samples (1 : 250 in FBS-PBS) were added and incubated for 1 h at room temperature. Plates were washed, and 100  $\mu$ l of peroxidase-conjugated goat anti-human IgM (1 : 1000) or IgG (1 : 6000), or anti-mouse IgG secondary antibody (Dako)

in FBS-PBS were added for 1 h at room temperature with gentle shaking. Plates were washed, and specific antibody binding was visualized by the addition of 100  $\mu$ l of *O*-phenyldiamine (Sigma) in 0.02 M acetate buffer with 0.0004% H<sub>2</sub>O<sub>2</sub> to each well. After 20 min, the reaction was stopped with 50  $\mu$ l of 1 M HCl, and the plates were read at 492 nm with a reference filter at 405 nm. All samples were analysed in duplicate, and the optical densities of the background wells were subtracted from the test samples. As a control, we have used the D-110 antibody (diluted 1 : 20 000 and 1 : 40 000). Both, the inter- and intra-assay variations were <15%.

To test for a possible cross-reactivity between human sera and D-110 antibody, plates were coated with recombinant CDV nucleocapsid protein and blocked with PBS-FBS as described above. The plates were then incubated with human sera (1 : 100 in PBS-FBS) for 1 h at room temperature, washed and incubated with D-110 antibody (1 : 20 000 in PBS-FBS) for 1 h at room temperature. After washing, bound antibodies were visualized with peroxidase-conjugated goat anti-mouse IgG secondary antibody as described above. Cross-reactivity was measured as the percentage inhibition of D-110 antibody binding. Alternatively, plates were incubated with D-110 antibody (1 : 20 000 in PBS-FBS) for 1 h at room temperature, washed, incubated with human sera (1 : 250 in PBS-FBS) for 1 h at room temperature, and bound antibodies were visualized with peroxidase-conjugated goat anti-human IgM or IgG secondary antibody as described above. In this experiment, cross-reactivity was measured as the percentage inhibition of human serum binding

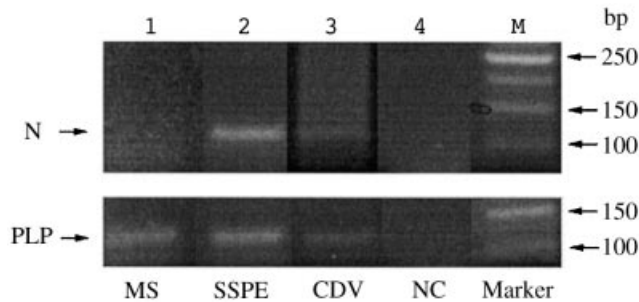
### Statistical evaluation

Statistical analysis (means, standard deviations and medians), significance of group differences, linear regressions and Pearson correlations were evaluated using the GraphPad Prism statistical analysis program (GraphPad Software, San Diego, CA, USA). Distribution of groups was analysed by the Kolmogorov-Smirnov test. Between-group comparisons of normally distributed data were then made using parametric one-way ANOVA (analysis of variance) with the Bonferroni post-test.  $P$  values <0.05 were considered as statistically significant.

## Results

### *D-110 epitope is expressed in a distinct subtype of actively demyelinating multiple sclerosis lesions*

Actively demyelinating lesions in multiple sclerosis patients can be separated into four distinctly different patterns (Lucchinetti *et al.*, 2000). These patterns of demyelination were the same in all active lesions within an autopsy of a single patient, but differed in essential aspects of immunopathology between different multiple sclerosis patients.



**Fig. 3** RT-PCR for the detection of nucleocapsid protein (N)- and PLP-specific RNAs isolated from paraffin-embedded formalin-fixed brain tissue. PCR-derived fragments were separated on a 3% agarose gel. In the upper panel, the 128 bp N-specific PCR product was detected in RNA isolated from subacute sclerosing panencephalitis and CDV, but not in cases of multiple sclerosis material and negative control (NC). The lower panel shows the PCR products of a PLP specific RT-PCR. This reaction was performed with the same RNA as the N-specific RT-PCR. The sizes of the molecular size markers (M) are indicated in base pairs (bp) on the left.

Pattern I lesions are well demarcated and centred by small vessels with focal infiltrates of T lymphocytes and macrophages. Pattern II lesions are similar to those described above, but show in addition deposition of immunoglobulins and activated complement (C9neo antigen) at the sites of active myelin destruction (Storch *et al.*, 1998; Genain *et al.*, 1999). Pattern III lesions reveal ill-defined contours. The initial lesions are characterized by a distal oligodendroglialopathy, defined by extensive or complete loss of myelin-associated glycoprotein in the zones of active myelin destruction, in comparison with other myelin proteins, such as PLP or myelin oligodendrocyte glycoprotein (Itayama *et al.*, 1980). The subsequent demyelinating process is associated with a high incidence of oligodendrocytes, showing the typical morphological features of apoptosis. Pattern IV lesions are sharply demarcated, with similar T cell and macrophage distribution to that described for pattern I. Deposition of immunoglobulins and activated complement is absent; there is, however, a high incidence of oligodendrocytes with DNA fragmentation in a narrow zone just outside the area of active myelin destruction (Lucchinetti *et al.*, 1996).

Massive expression of the D-110 epitope was found in all actively demyelinating pattern III lesions (Figs 1 and 2). The expression was most pronounced at sites of active myelin destruction, and much lower in the inactive centre of the plaques. Only exceptional immunoreactive cells were found in the periplaque and distant white and grey matter. Expression of the D-110 epitope was not restricted to a specific cell type, but was found in oligodendrocytes, astrocytes, microglia cells and macrophages (Fig. 1). Oligodendrocytes, however, were stained most intensely compared with other cells. In contrast to pattern III lesions, actively demyelinating plaques following other patterns of

demyelination (patterns I, II and IV) showed only minor immunoreactivity with this antibody regardless of the stage of the demyelinating process, as defined by stringent criteria (Brück *et al.*, 1995; Figs 1 and 2). Similarly, cells with D-110 immunoreactivity were only encountered exceptionally in the normal brain of control patients. As a positive control, massive expression of this epitope was found in similar cell types in dog brains infected with CDV.

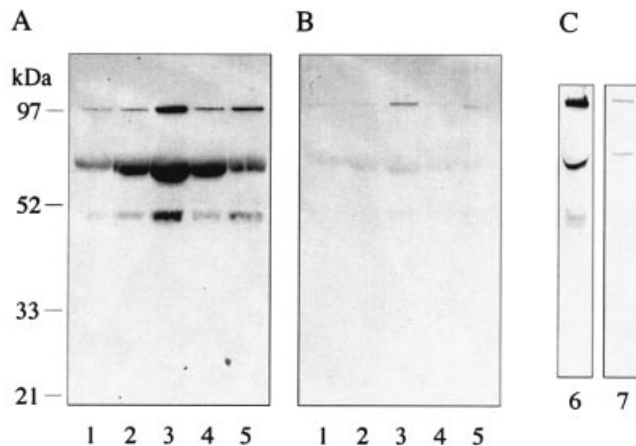
### ***D-110 expression in the multiple sclerosis brain is not due to infection with CDV or a related paramyxovirus***

The observed expression of the D-110 epitope could reflect infection of the brain of a subset of multiple sclerosis patients with CDV or a related paramyxovirus. We therefore screened the tissue by immunocytochemistry for other CDV antigens or by *in situ* hybridization for CDV RNA sequences. As a positive control, formalin-fixed, paraffin-embedded brain material from dogs with canine distemper encephalomyelitis was used. In contrast to very strong positive signals in the CDV-infected dog brains, we found no reactivity with any of the other antibodies recognizing the N, H and M protein or probes complementary to N and P of CDV in any of the multiple sclerosis sections (data not shown).

To investigate this question further, we developed PCR primers intended to recognize a broad variety of different paramyxovirus strains. These PCR primers were located in a highly conserved region of the nucleocapsid protein and were able to amplify the paramyxovirus sequence when RT-PCR was performed from total RNA extracted from paraffin-embedded formalin-fixed sections of CDV-infected dog brain or a patient with subacute sclerosing panencephalitis. However, in spite of positive signals for sequences of the myelin protein PLP as an RNA quality control, we found no amplified paramyxovirus sequence in RNA isolated from multiple sclerosis brain tissue, which presented with intensive D-110 immunoreactivity, even when a boosted PCR was performed (Fig. 3).

### ***Expression of the D-110 epitope in diseases other than multiple sclerosis***

We then investigated whether the expression of the D-110 epitope is specific for this subset of multiple sclerosis patients, or whether it can also be found in other brain diseases. The most consistent expression pattern of D-110 antigen was found in ischaemic brain lesions during the early stage of active tissue destruction (Figs 1 and 2). There, D-110 reactivity was concentrated mainly at the lesion border and in a small rim of perilesional white matter, while the necrotic centre of the infarct revealed only a few immunoreactive cells. D-110 reactivity was present mainly in oligodendrocytes and astrocytes as well as in some macrophages and activated microglia. In

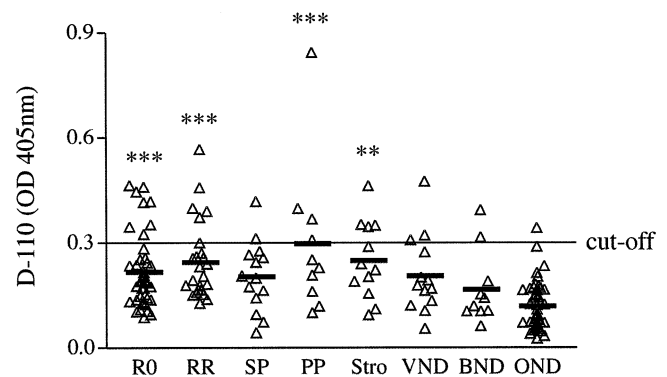


**Fig. 4** (A) Reactivity of D-110 antibody with the CSF of patients with OND (lane 1), VND (lane 2), stroke (lane 3), first attack multiple sclerosis (lane 4) and primary proliferating multiple sclerosis (lane 5) in western blots. CSF (25  $\mu$ l/lane) was separated by SDS-PAGE, transferred to nitrocellulose membranes and incubated with D-110. Reactive proteins were visualized with enhanced chemiluminescence. (B) Dephosphorylation of this blot abolished all signals. (C) The CSF of two multiple sclerosis patients, in whom a diagnostic brain biopsy was performed: lane 6, patient with pattern III demyelinating lesion; lane 7, patient with a pattern II demyelinating plaque. Molecular weight standards (kDa) are shown on the left.

cortico-subcortical infarcts, D-110-reactive neurons were also found, mainly located at the lesional edges. D-110 expression was highest during the first week after onset of stroke and declined rapidly thereafter. No expression was noted in infarcts older than 6 weeks (Fig. 2). No D-110 immunoreactivity was found in myocardial infarcts, irrespective of the stage of lesion development.

In addition, we found a variable expression of this antigen in virus-induced brain diseases (Fig. 2). An expression comparable in cell numbers and staining intensity with that in pattern III multiple sclerosis lesions was found in some cases of progressive multifocal leucoencephalopathy, and in cytomegalovirus or herpes simplex virus encephalitis. There, the expression, as in multiple sclerosis, was restricted to areas which were in the active stage of tissue damage. Different cells, including oligodendrocytes, astrocytes, microglia cells and macrophages, were stained. However, D-110 expression was only found in a subset of cases, while in others no staining was observed in spite of local expression of the different disease-related virus antigens or active tissue destruction.

The common denominator of all lesions in stroke and encephalitis patients with high expression of the D-110 epitope was a pattern of myelin and oligodendrocyte damage, closely reflecting that in multiple sclerosis pattern III. All lesions showed a preferential loss of myelin-associated protein and cyclic nucleotide phosphodiesterase, as well as nuclear condensation and fragmentation in oligodendrocytes.



**Fig. 5** Individual levels of D-110 immunoreactivity measured by ELISA in the CSF of patients with multiple sclerosis, first attack (R0), relapsing–remitting (RR), secondary chronic progressive (SP) and primary chronic progressive (PP) disease course; stroke (Stro), viral and bacterial neurological disease (VND and BND) and other neurological diseases (OND). Between-group comparisons of normally distributed data were made using parametric ANOVA with the Bonferroni post-test. Significant differences from OND are shown as \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . A ‘cut-off’ value (mean  $\pm$  2.5 SD; 0.3 OD units) was calculated from the OND group.

### D-110-reactive protein is liberated into the CSF in brain disease

Analysis of the CSF of patients with multiple sclerosis and other neurological diseases by western blot revealed at least three immunoreactive bands of 115, 70 and 50 kDa (Fig. 4A). We failed to detect any D-110 immunoreactivity in serum.

Next we developed an ELISA system to quantify CSF antigens recognized by D-110. As can be seen in Fig. 5, D-110 values were lowest in patients with ‘normal CSF’ (OND); only two patients with headaches showed higher D-110 values. The first attack, relapsing–remitting and primary progressive clinical multiple sclerosis subgroups (but not secondary chronic progressive) and stroke patients had significantly higher CSF D-110 values than the OND controls.

From the OND group, we calculated a ‘cut-off’ value (mean  $\pm$  2.5 SD; 0.3 OD units). Eight out of 43 first attack patients (19%), six out of 25 relapsing–remitting patients (24%), two out of 14 secondary chronic progressive patients (14%), four out of 10 primary progressive patients (40%), five out of 12 stroke patients (42%), three out of 13 VND patients (23%), two out of 10 BND patients (20%) and two out of 46 OND patients (4%) had D-110 values above this cut-off value ( $>0.3$ ).

Using recombinant CDV nucleocapsid protein as an antigen, we screened the sera of our patients and healthy controls for the presence of anti-CDV nucleocapsid IgM and IgG antibodies. These antibodies were, as would be expected owing to the sequence homology of this protein with the nucleocapsid protein of other paramyxoviruses (such as measles and parainfluenza virus), present at equal titres in all



groups analysed ( $P > 0.05$ ). Furthermore, we found no correlation between serum IgM or IgG antibodies and CSF D-110 antigen levels. We have also investigated whether serum antibodies to CDV nucleocapsid protein are cross-reactive with D-110 antibody by ELISA using pre-incubation of this antigen with sera and/or D-110 antibody. However, we found no significant cross-reaction between D-110 antibody and human sera (<10%), indicating that human serum antibodies are not reactive with the epitope recognized by D-110 antibody.

A retrospective analysis of the clinical course of patients did not reveal significant correlates between CSF D-110 levels and type of disease (relapsing–remitting, primary or secondary progressive), severity of disease, conversion to clinically definite multiple sclerosis, annual relapse rate or disease progression.

### ***Direct correlation between CSF values of D-110 with neuropathology***

In four multiple sclerosis cases included in this series, both a brain biopsy and CSF samples were available. One case, which was classified by neuropathology as pattern III demyelination, showed significantly elevated levels of the D-110 protein in the CSF. The other three cases, showing a pattern II lesion in pathology, only revealed CSF levels of the D-110 epitope comparable with controls (Fig. 4C, Table 2). Repeated CSF analysis during the disease course in the multiple sclerosis patient with pattern III lesions showed elevated D-110 values during the active stage of the disease, while the levels declined below the cut-off level when the patient entered remission, defined both by clinical parameters and the disappearance of lesion enhancement in MRI.

### ***D-110 recognizes a phosphorylation-dependent epitope, related to brain hypoxia***

Pre-treatment of brain sections with alkaline phosphatase prior to immunocytochemistry completely abolished D-110 immunoreactivity. To confirm that the antibody recognizes a phosphorylated epitope, we incubated a western blot identical to that shown in Fig. 4A with alkaline phosphatase. As can be seen in Fig. 4B, this treatment abolished the binding of D-110 to CSF proteins, thus confirming the findings from immunohistochemistry. All brain lesions with D-110 immunoreactivity, irrespective of their primary ischaemic or inflammatory nature, also showed expression of HIF-1 $\alpha$  within nuclei of glial cells, neurons, macrophages or endothelial cells (Fig. 2). There was a significant correlation between D-110 and HIF-1 $\alpha$  expression within the lesions (correlation coefficient: 0.72;  $P < 0.0001$ ). However, no HIF-1 $\alpha$ -immunoreactive material was detected in the CSF.

## **Discussion**

This study was designed originally to search for a possible involvement of CDV as a trigger for multiple sclerosis. Indirect evidence for an association between CDV infection and multiple sclerosis comes from epidemiological studies as well as from the increased incidence of CDV reactivity in CSF immunoglobulins of patients (Cook *et al.*, 1995; Rohowsky-Kochan *et al.*, 1995). As we had access to a large sample of multiple sclerosis biopsies and autopsies from the earliest stages of the disease, it was thus obvious to search for CDV antigen and/or sequences by immunocytochemistry and *in situ* hybridization. Indeed, we found a very pronounced immunoreactivity in a particular subset of multiple sclerosis patients with a monoclonal antibody against CDV nucleocapsid protein. Patients with the highest expression of this antigen in the lesions all followed the pathological pattern III, which in several respects, such as loss of myelin-associated glycoprotein and oligodendrocyte apoptosis, mimics changes previously described in virus-induced demyelinating disease (Itoyama *et al.*, 1982). Nevertheless, a detailed analysis of antigens and RNA sequences from other parts of CDV revealed no further positive results and thus rules out that the expression of the D-110 antigen indeed reflects CDV infection of the tissue. Furthermore, PCR analysis using primers to regions conserved within the paramyxovirus family was negative. Thus, even the possibility that D-110 recognizes a cross-reactive sequence within another member of the paramyxovirus family seems unlikely. These results are underscored further by the fact that this epitope can be found in a variety of other neuropathological conditions, such as different virus-induced brain diseases or acute stroke. All these data strongly indicate that the D-110 antibody recognizes a cross-reactive epitope on an endogenous protein that is upregulated in the CNS under special pathological conditions.

Besides in the distinct subtype of multiple sclerosis lesions, the most pronounced expression of D-110 antigen was found in some virus-induced lesions and in acute ischaemia. All lesions with high D-110 expression, irrespective of the cause of the primary disease, had in common a similar pattern of myelin and tissue destruction, characterized by loss of myelin-associated glycoprotein, and nuclear condensation and fragmentation in oligodendrocytes, suggesting a common pathogenetic pathway of tissue injury. In addition, all these lesions showed a massive expression and nuclear translocation of HIF-1 $\alpha$ . These data thus suggest that a hypoxia-like metabolic injury may be involved in tissue damage not only in stroke but also in inflammatory brain lesions.

In inflammatory conditions, hypoxia-like tissue damage could result from ischaemia, induced by a disturbance of the microcirculation due to massive focal oedema in areas which are restricted in swelling by tight connective tissue or bony constrains (Prineas and McDonald, 1997). Furthermore, thrombotic occlusion of small inflamed vessels can occur both in virus encephalitis (Esiri and Kennedy, 1997) and in

multiple sclerosis (Putnam, 1933; Wakefield *et al.*, 1994). Alternatively, key molecules involved in tissue injury, such as for instance excitotoxins (Oka *et al.*, 1993; McDonald *et al.*, 1998) as well as reactive oxygen and nitrogen intermediates (Smith *et al.*, 1999), may be shared in ischaemic as well as inflammatory brain lesions. In particular, peroxynitrite may impair mitochondrial function in multiple sclerosis lesions (Bolanos *et al.*, 1997; Heales *et al.*, 1999; Lu *et al.*, 2000) leading, as in true ischaemia, to cellular energy deficiency and nuclear translocation of HIF-1 $\alpha$  (Kimura *et al.*, 2000; Semenza, 2000).

So far, it has not been determined which protein in the human CNS is recognized by the D-110 antibody. This antibody selectively detects a non-phosphorylated CDV nucleocapsid epitope (A. Zurbriggen, unpublished) and a phosphorylated endogenous brain-specific epitope. Several examples exist where an antibody cross-reacts with a phosphorylated epitope in one protein and a non-phosphorylated epitope in a different protein. In these cases, the phosphorylated amino acid is substituted by a charged amino acid such as glutamic acid or aspartic acid (Al-Hillawi *et al.*, 1998). Since in human brain the antibody reacts with a phosphorylated epitope, the most likely candidate would be an endogenous brain protein, selectively phosphorylated in a stress response to ischaemia or a related metabolic injury.

Irrespective of the nature of this antigen, its liberation into the CSF may have practical consequences. Although not specific for multiple sclerosis, it may allow the identification of a subset of patients with a pathogenetically distinct pattern of tissue injury. Within the population of patients with multiple sclerosis or virus encephalitis, it may recognize those in which hypoxia-like tissue injury contributes to the development of the lesions. Such a paraclinical marker is the prerequisite for the future design of subtype-specific therapeutic strategies in the respective patients. Our preliminary data suggest that the D-110 epitope may indeed fulfil the criteria for such a suitable paraclinical marker. In pathology, the expression of this marker is associated with the respective pattern of tissue injury with a very high degree of specificity. In the CSF of multiple sclerosis patients, this protein is present on average in 17% of the patients, a percentage which closely matches the incidence of cases with pattern III lesions found in pathological investigations (Lucchinetti *et al.*, 2000). In addition, in the four cases where both a biopsy and CSF sample were available, the CSF levels of D-110 immunoreactivity matched the immunopathological pattern of the lesions. Thus, in pathology, D-110 is apparently a reliable marker for identifying a defined subtype of inflammatory brain lesions. Although supported by indirect evidence, our data so far are too preliminary to answer the question of whether the levels of D-110 in the CSF accurately reflect the metabolic alterations within the lesions. Further direct correlations between pathology and CSF values of D-110 will be necessary to determine the reliability of this tool for diagnosis and subtyping of inflammatory brain diseases.

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