Identification of DAGLA as an autoantibody target in cerebellar ataxia


ABSTRACT

Background We aimed to investigate the clinical, imaging and fluid biomarker characteristics in patients with diacylglycerol lipase alpha (DAGLA)-autoantibody-associated cerebellar ataxia.

Methods Serum and cerebrospinal fluid (CSF) samples from four index patients were subjected to comprehensive autoantibody screening by indirect immunofluorescence assay (IIFA). Immunoprecipitation, mass spectrometry and recombinant protein assays were used to identify the autoantigen. Sera from 101 patients with various neurological symptoms and a similar tissue staining pattern as the index patient samples, and 102 healthy donors were analysed in recombinant cell-based IIFA (RC-IIFA) with the identified protein. Epitope characterisation of all positive samples was performed via ELISA, immunoblot, immunoprecipitation and RC-IIFA using different DAGLA fragments.

Results All index patients were relatively young (age: 18–34) and suffered from pronounced gait ataxia, dysarthria and visual impairments. Parachinal hallmarks in early-stage disease were inflammatory CSF changes and cerebellar cortex hyperintensity in MRI. Severe cerebellar atrophy developed in three of four patients within 6 months. All patient samples showed the same unclassified IgG reaction with the cerebellar molecular layer. DAGLA was identified as the target antigen and confirmed by competitive inhibition experiments and DAGLA-specific RC-IIFA. In RC-IIFA, serum reactivity against DAGLA was also found in 17/101 disease controls, including patients with different clinical phenotypes than the one of the index patients, and in 1/102 healthy donors. Epitope characterisation revealed that 17/18 anti-DAGLA-positive control sera reacted with a C-terminal intracellular DAGLA 583–1042 fragment, while the CSF samples of the index patients targeted a conformational epitope between amino acid 1 and 157.

Conclusions We propose that anti-DAGLA autoantibodies detected in CSF, with a characteristic tissue IIFA pattern, represent novel biomarkers for rapidly progressive cerebellitis.

WHAT IS ALREADY KNOWN ON THIS TOPIC

Autoimmune cerebellar ataxias are characterised by an impaired ability to coordinate muscle movement and can lead to strong disabilities. Cerebellar autoantibodies serve as useful biomarkers for rapid disease diagnosis, but recurrently, severe neurological disorders of potential autoimmune origin are observed in which the autoantibody target has not been identified.

WHAT THIS STUDY ADDS

In this study, we identified diacylglycerol lipase alpha (DAGLA) as the autoantibody target in four young patients with a rapid progressive disabling cerebellar ataxia. The autoantibodies of these patients react predominantly with the molecular layer on cerebellar tissue sections and target a conformational epitope between amino acid 1 and 157. In clinical practice, anti-DAGLA autoantibodies detected in CSF with a characteristic tissue IIFA pattern should be considered in the diagnosis of autoantibody-associated cerebellitis.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

The clinical course with high inflammatory changes at disease onset and a rapid relapse after cessation of immunotherapy raise the question whether earlier diagnosis, followed by more aggressive and prolonged immunotherapy could inhibit dramatic disease progression.

INTRODUCTION

Cerebellar ataxia can be caused by different aetiologies including genetic, infectious or neurodegenerative diseases, toxin exposure or autoimmune-mediated processes. Independently of the cause, the destruction of cerebellar neurons or loss of cell function leads to similar symptoms, including difficulty with speech and coordination, imbalance and gait disorders. However, therapy strategy and response as well as prognosis depend on the disease aetiology. Therefore, rapid and precise diagnosis is necessary to facilitate early and suitable therapy before irreversible damage on cerebellar structures occurs and therapeutic opportunities...
vanish. Autoantibodies serve as valuable markers for the diagnosis of autoimmune cerebellar ataxias.1,2 Some of these autoantibodies (eg, anti-Hu, anti-Yo, anti-Ri, anti-CV2, anti-Ma2, anti-amphiphysin, anti-SOX1, anti-Kelch-like protein 11 and anti-Regulator of G-protein signalling 8) target intracellular antigens. Their detection is a strong indication of an underlying neoplasia which is assumed to cause autoantibody production.3–6 In these paraneoplastic syndromes, antineuronal antibodies react with antigens expressed on both tumour and brain tissue.7 In other cases, autoantibody production is largely unknown. However, viral infections, or vaccinations are discussed as triggers for autoimmune responses in some cases.15–17

Despite the growing number of characterised neuronal autoantigens, autoantibodies targeting unknown cerebellar target proteins are still detected frequently in indirect immunofluorescence assays (IFAs) using cerebellar tissue.18

In this study, we describe four patients with severe cerebellar ataxia and similar IgG reactivity on cerebellar tissues. Autoantibodies targeting the novel cerebellar autoantigen sn1-specific diacylglycerol lipase alpha (DAGLA) were detected in CSF samples of all patients.

MATERIALS AND METHODS

Patients

Between April 2022 and February 2023, the four index patients were diagnosed and treated at the departments of neurology of the contributing hospitals. The Clinical Immunological Laboratory Prof. Dr. med. W. Stöcker, Lübeck (Germany) received the samples for the purpose of autoantibody testing. Subsequently, they were anonymised and provided to the authors. Written informed consent to the publication of all clinical information was obtained from all patients whose clinical data are reported in this study. An approval from the Hannover Medical School institutional review board was received (2481-2014).

The analysed panels included sera of 102 healthy donors (healthy controls, HC), 48 CSF samples that were negative in IIFA using brain tissue and 101 sera from patients with a similar staining pattern on cerebellum as the index patients, for which neuronal autoantibody testing was initiated by the treating physician based on the clinical presentation of a suspected autoantibody-associated neurological disease (disease cohort (DC)).

Antibody-specific index (AI)

The calculation of the DAGLA CSF/serum antibody-specific index (AI) was performed according to the formula Qspec/ QlG. In case of intrathecal IgG production (QlG>Qlim), as indicated by the Reiber diagram, the AI was calculated as Qspec/ Qlim. The cut-off value for a positive AI was set to values >4 based on the titre scale.19

Indirect immunofluorescence assay

IIFA using slides with a biochip array of brain tissue cryosections combined with recombinant human embryonic kidney (HEK293) cells separately expressing 35 different brain antigens (online supplemental material) was incubated as described previously.20 Additionally, IIFA was performed using recombinant acetone-fixed HEK293 cells expressing DAGLA, DAGLA 1–582, DAGLA 1–157 and empty vector-transfected HEK293 cells as control substrate.

IgG subclass determination was performed with serum of P1–4, HC29, DC1–5, 8, 9, 11, 12, 15 and IgG subclass-specific fluorescein isothiocyanate (FITC)-labelled mouse anti-human IgG (Sigma-Aldrich F0767, F4516, F4641, F9890, final dilution of 1:25).

For colocalisation assay, patient serum was incubated together with a polyclonal rabbit antibody against DAGLA (1:50, Sigma-Aldrich, HPA062497) in the first step, followed by incubation with anti-human IgG-Alexa488 and anti-rabbit IgG-Cy3 (1:400, Dianova).

Immunoprecipitation

The immunoprecipitation was performed with 200 µL tissue homogenate of rat brain or 50 µL DAGLA 1–582-His expressing HEK293 cells (50 million cells/mL) and 30 µL serum or 30 µL CSF in 500 µL solubilisation buffer.20 The supernatants were incubated with Protein G Dynabeads (ThermoFisher Scientific) containing 25 mmol/L dithiothreitol followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, NuPAGE, ThermoFisher Sc ientific) and Coomassie Brilliant Blue (G-250) (Merck) gel staining. Selected protein bands were analysed by mass spectrometry as described in online supplemental material. Alternatively, eluates were used for immunoblotting.

Immunoblot

Immunoprecipitated cerebellum lysate or HEK293-DAGLA 1–582-His, as well as HEK293-DAGLA cell lysates were subjected to immunoblotting as previously described.4 Membranes were incubated with sera or CSFs (dilution 1:200), rabbit anti-DAGLA antibody (1:2000, Sigma Aldrich, HPA062497) or mouse anti-His (1:2000, Merck, 70796-3) in 1:5 diluted sample buffer (EUROIMMUN AG) for 3 hour, followed by an incubation with anti-human-IgG-AP (1:10, EUROIMMUN AG), anti-rabbit IgG-AP (1:2000, Jackson Research) or anti-mouse IgG-AP (1:2000, Sigma-Aldrich).

Recombinant expression of full-length DAGLA and DAGLA fragments in HEK293 and E.coli

The cDNA encoding human DAGLA was obtained from Source BioScience UK Limited as clone IRATp9700E05140D. Cloning of full-length DAGLA, DAGLA 1–582, and DAGLA 1–157 into pTriEx-1 (Merck) or DAGLA fragments H8-GST-DAGLA 1–22, H8-GST-DAGLA 44–66, H8-GST-DAGLA 82–101, H8-GST-DAGLA 123–136, DAGLA 158–598 and DAGLA 583–1042 into a modified pET24d (Merck) plasmid vector and recombinant expression in HEK293 cells or E. coli is described in the online supplemental material.

Purification

Bacterial cells expressing DAGLA fragments were lysed, and proteins were solubilised in urea buffer and purified by Ni2+-affinity chromatography as previously described.4 The proteins were stored in aliquots at −80°C until further use.

ELISA

ELISA was performed as described previously.4 Briefly, 96-well plates were coated with 100 µL of the recombinant protein at a concentration of 2.5 µg/mL in phosphate buffered saline (PBS) for 2 hours at 25°C. CSF and serum samples were diluted 1:100.
and anti-His antibody 1:2000 (Sigma-Aldrich) in sample buffer (1% casein, 0.05% Tween 20 in PBS) and incubated for 30 min. Bound antibodies were detected with anti-mouse IgG-POD conjugate (Jackson Research) diluted 1:16000 in sample buffer or anti-human IgG-POD (undiluted, EUROIMMUN AG).

**RESULTS**

**Patients**

The four young index patients (18–34 years old; male:female 3:1) were hospitalised between 2022 and 2023. Their detailed individual disease courses can be found in the online supplemental results and in table 1. Except for patient 4 who had a history of refractory ulcerative colitis, all were previously healthy. Within weeks they developed a progressive cerebellar ataxia with midline accentuation characterised by trunk, stance and gait ataxia, dysarthria/anarthria and ocular motor dysfunction. Additionally, psychiatric examination revealed emotional lability in patients 2 and 3, developing into a severe psychosis over time in patient 3. The brain MRIs revealed T2 hyperintense signal changes in the cerebellar hemispheres with parenchymal swelling suggestive of cerebellitis (figure 1). Malignancy was excluded during the diagnostic work-up. CSF analysis revealed a significant lymphocytic pleocytosis (0.132–0.406*10^6 WBC/L). Anti-infective treatment was started and changed to high-dose steroids after microbiological and virological laboratory tests were negative. Autoimmune cerebellitis was suspected and by extensive autoantibody analysis anti-DAGLA antibodies were detected in serum and CSF of all patients with an autoantibody-specific index that proved an intrathecal synthesis (AI 59–434).

After high-dose steroid treatment, patient 1 was transferred to an inpatient rehabilitation programme. Within 7 weeks after onset ataxia and dysarthria progressed and the patient was unable to walk, so intravenous immunoglobulin (IVIG) treatment was started. Patients 2 and 3 were initially treated with IVIG in addition to the high-dose steroids. IVIG slightly improved symptoms in patients 1 and 2. Two of the patients were started on an oral steroid taper but relapsed on prednisolone 20 mg/day (patient 2) and 16 mg (patient 4) every other day. Subsequently, all patients were treated with plasma exchange or immunoadsorption resulting in a measurable improvement in patients 3 and 4. Patient 4 received 6 cycles of cyclophosphamide induction treatment. Maintenance immunosuppressive therapy was established with rituximab in the other three patients. In patient 2, this was done after the relapse on azathioprine and steroid tapering.

During follow-up CSF analyses, the pleocytosis regressed, except for a slight increase in cell count in patient 2 during the relapse. Patients 1, 2 and 4 showed a slow individual variable improvement of their cerebellar symptoms resulting in a reduced mRS in patients 1 and 4. Patient 3 worsened after temporary mild improvement with mRS 5 at last follow-up. However, all were still severely affected reflected by a cerebellar atrophy on subsequent brain MRIs.

**Characterisation of the patients’ autoantibodies**

In IIFA, CSFs and sera of patients 1–4 (P1–4) produced the same IgG staining patterns in the molecular layer of rat and monkey cerebellum cryosections. The autoantibodies bound exclusively to the dendrites of the Purkinje cells, whereas the somata remained unstained (figure 2A, B). Apart from the prominent reactivity with the cerebellum a weaker but clear staining of the hippocampus was observed on sagittal sections of murine whole brain and rat hippocampus (figure 2C–F). Comparable reactivities were observed by immunohistochemical staining of rat brain sections (online supplemental figure 1). None of the patient CSFs/sera was found positive by IIFA for a panel of 35 recombinantly expressed established neural autoantigens, including the known Purkinje-cell antigens.

**Identification of DAGLA as the neuronal target antigen**

Immunoprecipitations with CSF and serum of P2 and lyses of rat cerebellum were able to enrich a protein at approximately 100 kDa that was identified by mass spectrometry as DAGLA (UNIPROT acc. #Q5SYLM1, figure 3A, B). This result was confirmed in immunoblots with the immunoprecipitates and a monospecific anti-DAGLA rabbit antibody (figure 3C). IIFA with cerebellum sections using patient CSF and the anti-DAGLA rabbit antibody revealed an exact overlap of the molecular layer staining (figure 3D). Moreover, preincubation of the patients’ CSFs with HEK293 lysate containing recombinant DAGLA eliminated the tissue reactivity of the patients’ CSFs (figure 3E).

**Detection of anti-DAGLA autoantibodies by recombinant IIFA**

In recombinant cell-based IIFA (RC-IIFA) with HEK293 expressing DAGLA, patients’ CSFs and sera reacted strongly positive (IgG end titers CSF: P1–2 1:320, P3 1:3200, P4 1:1000; serum: P1 1:1000, P2 1:320, P3 1:3200, P4 1:32, for IgG subclasses see table 1), whereas control cells did not demonstrate any specific antibody binding (figure 4 and table 1). Of 48 tissue-IIFA-negative CSF samples, none showed a positive reaction in anti-DAGLA RC-IIFA. However, of 102 healthy control sera one reacted positively with the DAGLA expressing HEK293 cells (HC29, IgG end titre 1:1000, IgG1 subclass). Surprisingly, this serum displayed a similar tissue IIFA reactivity as the patient samples (online supplemental figure 2). We subsequently analysed 101 sera of patients suffering from various neurological aberrations (DC) with a tissue IIFA staining pattern on cerebellum similar to the patient samples in the anti-DAGLA RC-IIFA (online supplemental figure 2). These sera did not react with any of the 35 established neural autoantigens but anti-DAGLA autoantibodies could be detected in 17/101 of the patients by RC-IIFA (DC1–17, serum IgG end titers 1:100–1:1000, for IgG subclasses see online supplemental table 3). Corresponding CSF samples were available for five patients. Only one of these CSFs showed an anti-DAGLA-characteristic staining in tissue IIFA and was also positive in anti-DAGLA RC-IIFA (DC9, CSF IgG end titre 1:1000), two were negative in tissue IIFA and weakly positive in anti-DAGLA RC-IIFA (DC5, DC14, both CSF IgG end titre 1:1) and two were negative in tissue IIFA and anti-DAGLA RC-IIFA (DC10, DC15). The clinical data available for six of the anti-DAGLA-positive DC patients were diverse (DC8: migraine, DC9: cerebellitis, DC10: psychotic disorder, DC11: dementia, DC14: sensory neuropathy, DC15: epilepsy) and mostly different to the clinical phenotypes of the index patients (P1–4). Interestingly, the only patient (DC9) with positive CSF results in tissue and anti-DAGLA RC-IIFA suffered from cerebellitis like the index patients. Together, these results indicate that anti-DAGLA autoantibodies detected in serum only are most likely not a marker for a distinct disease but instead suggest the use of tissue-IIFA-positive CSF samples for anti-DAGLA autoantibody detection. Indeed, when calculating the AIs all four index patients had highly elevated AIs (range: 35–434),
Table 1 Clinical and paraclinical data of the four DAGLA IgG-positive index patients

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Main clinical features*</th>
<th>Diagnosis</th>
<th>Brain MRI (weeks after onset)</th>
<th>EEG</th>
<th>CSF findings (weeks after onset)</th>
<th>Anti-DAGLA IgG titre/subtype (weeks after onset)</th>
<th>Immunotherapy (response) (weeks after onset)</th>
<th>mRS progression and outcome (follow-up time)</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>Trunk, stance, and gait ataxia (inability to walk), dysarthria, double vision and dysdiadochokinesia, downbeat nystagmus</td>
<td>Cerebellar syndrome</td>
<td>Initial MRI (1) unremarkable</td>
<td>Intermittent bilateral frontotemporal slowing</td>
<td>Initial CSF (1) lymphocytic pleocytosis</td>
<td>Initial titre (3) CSF 1:320/IgG1 Serum 1:1000/IgG1 Al 35</td>
<td>Methylprednisolone 1 g/day for 5 days (clinical symptoms unchanged) (2)</td>
<td>mRS 4 at diagnosis (4 weeks after onset)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FU-MRI (2) cerebellitis</td>
<td></td>
<td>FU CSF (3) WBC 0.063*10^9 cells/L</td>
<td>FU CSF (7) WBC 0.007*10^9 cells/L</td>
<td>4 cycles of immunoadsorption (6)</td>
<td>mRS 5 at peak of disease (7 weeks after onset)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FU-MRI (7) cerebellitis</td>
<td></td>
<td>FU CSF (7) WBC 0.007*10^9 cells/L</td>
<td>FU CSF (24) WBC 0.001*10^9 cells/L</td>
<td>IVIG 120 g over 4 days (mild improvement) (7)</td>
<td>mRS 4 (10 month) speech improved, patient able to walk with crutches, but still restricted</td>
</tr>
<tr>
<td>#2</td>
<td>Transferase deficiency syndrome</td>
<td>Cerebellar syndrome</td>
<td>Initial MRI (1) unremarkable</td>
<td>Intermittent bilateral frontotemporal slowing</td>
<td>Initial CSF (1) lymphocytic pleocytosis</td>
<td>Initial titre (3) CSF 1:320/IgG1 Serum 1:1000/IgG1 Al 35</td>
<td>Methylprednisolone 1 g/day for 5 days (clinical symptoms unchanged) (2)</td>
<td>mRS 4 at diagnosis (4 weeks after onset)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FU-MRI (2) cerebellitis</td>
<td></td>
<td>FU CSF (3) WBC 0.063*10^9 cells/L</td>
<td>FU CSF (7) WBC 0.007*10^9 cells/L</td>
<td>4 cycles of immunoadsorption (6)</td>
<td>mRS 5 at peak of disease (7 weeks after onset)</td>
</tr>
<tr>
<td>#3</td>
<td>Trunk, stance, and gait ataxia (inability to walk), dysarthria, double vision and dysdiadochokinesia, downbeat nystagmus</td>
<td>Cerebellar syndrome</td>
<td>Initial MRI (1) unremarkable</td>
<td>Intermittent bilateral frontotemporal slowing</td>
<td>Initial CSF (1) lymphocytic pleocytosis</td>
<td>Initial titre (3) CSF 1:320/IgG1 Serum 1:1000/IgG1 Al 35</td>
<td>Methylprednisolone 1 g/day for 5 days (clinical symptoms unchanged) (2)</td>
<td>mRS 4 at diagnosis (4 weeks after onset)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FU-MRI (2) cerebellitis</td>
<td></td>
<td>FU CSF (3) WBC 0.063*10^9 cells/L</td>
<td>FU CSF (7) WBC 0.007*10^9 cells/L</td>
<td>4 cycles of immunoadsorption (6)</td>
<td>mRS 5 at peak of disease (7 weeks after onset)</td>
</tr>
<tr>
<td>#4</td>
<td>Trunk, stance, and gait ataxia (inability to walk), dysarthria, double vision and dysdiadochokinesia, downbeat nystagmus</td>
<td>Cerebellar syndrome</td>
<td>Initial MRI (1) unremarkable</td>
<td>Intermittent bilateral frontotemporal slowing</td>
<td>Initial CSF (1) lymphocytic pleocytosis</td>
<td>Initial titre (3) CSF 1:320/IgG1 Serum 1:1000/IgG1 Al 35</td>
<td>Methylprednisolone 1 g/day for 5 days (clinical symptoms unchanged) (2)</td>
<td>mRS 4 at diagnosis (4 weeks after onset)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FU-MRI (2) cerebellitis</td>
<td></td>
<td>FU CSF (3) WBC 0.063*10^9 cells/L</td>
<td>FU CSF (7) WBC 0.007*10^9 cells/L</td>
<td>4 cycles of immunoadsorption (6)</td>
<td>mRS 5 at peak of disease (7 weeks after onset)</td>
</tr>
</tbody>
</table>

Continued
<table>
<thead>
<tr>
<th>Patient #</th>
<th>Main clinical features*</th>
<th>Diagnosis</th>
<th>Brain MRI (weeks after onset)</th>
<th>EEG</th>
<th>CSF findings (weeks after onset)</th>
<th>Anti-DAGLA IgG titre/subtype (weeks after onset)</th>
<th>Immunotherapy (response) (weeks after onset)</th>
<th>mRS progression and outcome (follow-up time)</th>
</tr>
</thead>
<tbody>
<tr>
<td>#2</td>
<td>Beginning with headache, nausea, vomiting, blurry vision and intermittent slurred speech, fast progression of tetra-ataxia (inability to walk, could sit freely only for a short period of time), pronounced oculomotor dysfunction (double vision and lack of fixation), head tremor and anarthria, psychological anomalies (fluctuating avolition, anxiety and restlessness)</td>
<td>Cerebellar syndrome</td>
<td>Initial MRI (1) unremarkable</td>
<td>Normal Alpha EEG</td>
<td>Initial CSF (2) lymphocytic pleocytosis</td>
<td>Initial titre (2) CSF 1:320/IgG1 Serum 1:320/IgG1</td>
<td>Methylprednisolone 1 g/day for 3 days (insufficient improvement) (2) prednisolone 100 mg/day p.o. (2)</td>
<td>mRS 4 at diagnosis (2 weeks after onset)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FU-MR (2) cerebellitis</td>
<td></td>
<td>FU CSF (4)</td>
<td>CSF 1:100</td>
<td>IVIG 180 g over 5 days (minor improvement) (3)</td>
<td>mRS 5 at peak of disease (3 weeks after onset)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FU-MR (3) cerebellitis</td>
<td></td>
<td>FU CSF (10)</td>
<td>WBC 0.032*10^9 cells/L</td>
<td>Prednisolone taper 80 mg/day (5)</td>
<td>mRS 4 (10 months) persisting cognitive impairment and (tetra-) ataxia, ability to stand could be restored, but essentially wheelchair users</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FU-MR (18) cerebellar atrophy</td>
<td></td>
<td>FU CSF (16)</td>
<td>WBC 0.022*10^9 cells/L</td>
<td>Prednisolone reduction 20 mg/day (10) (relapse) (13)</td>
<td>Prednisolone 100 mg/day p.o. (18)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Plasma exchange (stabilisation) (16)</td>
<td>Rituximab (20) in exchange for AZA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Prednisolone 100 mg/day p.o. (18)</td>
<td>Prednisolone 40 mg/day p.o. (25)</td>
<td></td>
</tr>
</tbody>
</table>
**Table 1**

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Main clinical features*</th>
<th>Diagnosis</th>
<th>Brain MRI (weeks after onset)</th>
<th>EEG</th>
<th>CSF findings (weeks after onset)</th>
<th>Anti-DAGLA IgG titre/subtype (weeks after onset)</th>
<th>Immunotherapy (response) (weeks after onset)</th>
<th>mRS progression and outcome (follow-up time)</th>
</tr>
</thead>
<tbody>
<tr>
<td>#3</td>
<td>Tetra-ataxia, abasia, impaired saccadic eye movements, progressive dysarthria/later anaesthesia with slow speech and hypophonia, dysphagia, depression, emotional instability, severe psychosis</td>
<td>Cerebellitis encephalitis</td>
<td>Initial MRI (2) cerebellitis and leptomeningitis</td>
<td>Generalised intermittent rhythmic delta activity (IRDA)</td>
<td>Initial CSF (1) lymphocytic pleocytosis</td>
<td>Initial titer (4) CSF: 1:3200/ IgG2&gt; IgG3 Serum 1:3200/ IgG1, IgG2 Al 120</td>
<td>Methylprednisolone 1 g/day for 5 days (2)</td>
<td>mRS 4 at diagnosis (6 weeks after onset)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FU MRI (9) decreasing leptomeningeal enhancement</td>
<td></td>
<td>WBC 0.159*10^9 cells/L</td>
<td>FU (19) CSF: 1:100 Serum: 1:10</td>
<td>IVIG 200 g over for 5 days (3)</td>
<td>mRS 5 at peak of disease (4 month after onset)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FU MRI (16) cerebellar atrophy</td>
<td></td>
<td>WBC 0.054*10^9 cells/L</td>
<td>Prednisolone 80 mg/day (7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FU CSF (19)</td>
<td></td>
<td>WBC 0 cells/L</td>
<td>Rituximab (10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FU CSF (19)</td>
<td></td>
<td>WBC 0 cells/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FU CSF (19)</td>
<td></td>
<td>WBC 0 cells/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#4</td>
<td>Headache, subacute gait ataxia, double vision due to bilateral abduction paresis, ataxia left hemisphericus, dysarthria, downbeat nystagmus</td>
<td>Rhomb encephalitis</td>
<td>Initial MRI (1.5) cerebellitis (hyperintense and swollen cerebellum)</td>
<td>EEG diffusely increased theta activity</td>
<td>Initial CSF (1.5) lymphocytic pleocytosis</td>
<td>Initial titer (3) CSF: 1:1000 Serum: 1:32</td>
<td>Methylprednisolone 1 g/day for 5 days (2)</td>
<td>mRS 4 at diagnosis (3 weeks after onset)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MRI (8) decreasing oedema</td>
<td></td>
<td>WBC 0.132*10^9 cells/L</td>
<td>FU (8) CSF: 1:100/ IgG2 Serum: 1:320/ IgG2 Al 59</td>
<td>Prednisolone 16 mg (EOD) (relapse) (8)</td>
<td>mRS 5 at peak of disease (8 weeks after onset)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FU MRI (8)</td>
<td></td>
<td>TP 44 mg/dL</td>
<td>5 cycles of plasma exchanges (slight improvement) (3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FU CSF (8)</td>
<td></td>
<td>OCB pos. (type 2)</td>
<td>Prednisolone 16 mg (EOD) (relapse) (8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FU CSF (8)</td>
<td></td>
<td>WBC 0.008*10^9 cells/L</td>
<td>5 cycles of plasma exchanges (8)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*In none of the patients an infection or a malignancy could be detected.

AI, antibody index; DAGLA, diacylglycerol lipase alpha; FU, follow-up; IVIG, intravenous immunoglobulin; mRS, Modified Rankin Score; OCB, oligoclonal bands; TP, cerebrospinal fluid total protein; WBC, white blood cells.
Neuro-inflammation

given a cut-off value for a positive AI > 4, proving an in-trathecal autoantibody production.19

Epitope mapping with DAGLA fragments
Because of the unexpected detection of high-titre anti-DAGLA autoantibodies in one healthy control serum and in sera of patients with diverse clinical phenotypes, we hypothesised that anti-DAGLA autoantibodies detected in CSF and serum samples of the cerebellitis patients (P1–4 and DC9) might recognise different epitopes than the anti-DAGLA autoantibodies detected in HC or DC sera with different phenotypes. To prove this hypothesis, six different fragments of the human DAGLA protein (amino acid 1–22, 44–60, 82–101, 123–136, 158–598, 583–1042) displaying all extracellular and intracellular sequence regions without transmembrane domains were recombinantly expressed in E. coli, purified and used in ELISA with CSF and serum.

Figure 1 Transverse T2 sections of the infratentorial brain (T1 for patient 3 and fluid-attenuated inversion recovery (FLAIR) for patient 4 at 18–28 weeks). At onset, T2-hyperintense signal changes in the cerebellar hemispheres were present in all patients (arrows). At follow-up, patients 1–3 showed a marked cerebellar atrophy (open arrows), whereas patient 4 showed only a mild atrophy.


Figure 2  Indirect immunofluorescence assays with brain tissues. Rat cerebellum (A), primate cerebellum (B), rat hippocampus (C), sagittal sections of murine whole brain (D), murine cerebellum (E) or murine hippocampus (F) permeabilised cryosections incubated with CSF of patient 1 followed by an incubation with Alexa488-labelled anti-human IgG. Nuclei were counterstained by incubation with TO-PRO-3 iodide or 4',6-diamidino-2-phenylindole (DAPI). A dense fine-speckled staining of the cerebellar molecular (A, B) and a weaker staining of the hippocampal molecular layer (C) were observed. On murine whole brain sections the patient CSF reacted mainly with the molecular layer of the cerebellum and also showed weaker reactivity against the hippocampus. No obvious reactivity against other parts of the brain was observed (scale bar A–C: 100 µm, E, F: 500 µm).
Neuro-inflammation

in the early stage of the disease were an inflammatory altered CSF with intrathecal autoantibody synthesis and a brain MRI with cerebellar cortex T2-hyperintensity and swelling. Next to the impaired cerebellar functions, mild impairment of declarative memory functions (P1), psychological anomalies (P2) and transitory (extra)pyramidal signs (P4) were observed. P3 developed severe psychosis, which due to its extreme extent appears beyond the scope of a cerebellar cognitive affective syndrome.

In agreement with the cerebellar and hippocampal disturbances, the patients’ autoantibodies showed predominant reactivity with these parts of the brain. It was previously described that DAGLA localises as multipass membrane protein on the surface of postsynaptic dendritic spines of cerebellar Purkinje cells and hippocampal neurons. There, it hydrolyses arachidonic acid-esterified diacylglycerols to produce the lipid messenger, 2-arachidonoylglycerol. This most abundant endocannabinoid influences synaptic signalling, axonal growth and neurogenesis. DAGLA knockout mice were characterised by loss of retrograde synaptic suppression, enhanced anxiety-like behaviours, as well as hypophagia with spontaneous seizures and decreased long-term survival. DAGLA gene duplication might be responsible for the development of dominantly inherited spinocerebellar ataxia 20 (SCA20). Similar to the clinical phenotype of anti-DAGLA-CSF-positive patients, SCA20...
is characterised by progressive ataxia, dysarthria, impaired eye movements, and cerebellar atrophy. In SCA20 it is assumed that a duplication of genomic DNA including DAGLA is responsible for the disease phenotype. However, it is not clear whether this leads to an increased amount of DAGLA protein levels or disturbed DAGLA activity. With respect to this, it would be interesting to unravel whether anti-DAGLA autoantibodies can act directly pathogenic, leading to inhibition or activation of protein function, if complement-mediated toxicity is triggered or T-cell mediated processes are involved. Further knowledge on anti-DAGLA pathophysiology might also help to find optimal therapeutic strategies.

Since high titers of anti-DAGLA autoantibodies were detected in CSF samples of our patients and other causes of the disease were excluded, immunotherapy was initiated in all four patients to a different extent. In general, treatment response was moderate, and severe impairments persisted in all patients. Initial steroid therapy had no or insufficient effects on clinical symptom progression, although CSF pleocytosis normalised and MRI inflammatory changes regressed. Immunoabsorption (P1), plasma exchange (P2, 3, 4) and IVIG treatment (P1, 2) led to an improvement, which was only temporary in three cases (P2-4).

Even though a very limited response steroids was observed in the early phase, two of the reported patients experienced a relapse under ongoing steroid tapering (P2, P4). While in patient 3 emotional liability possibly attributed to the cerebellar cognitive affective syndrome (CCAS), patient 1 had persisting deficits of declarative memory function, which are considered to be a red flag for non-cerebellar involvement. Patients 1–3 developed a pronounced cerebellar atrophy within half a year. Patient 4, who was treated with cyclophosphamide with respect to the outcome of the other patients, developed a mild cerebellar atrophy until 9 month follow-up and was able to walk a few metres unsupported. In patient 1 FDG PET at this phase revealed marked cerebellar hypometabolism. In all cases, anti-DAGLA titers in CSF were still high (IgG titre at least 1:100) after immunotherapy (table 1).

Based on these observations and the high inflammatory changes at disease onset it might be possible that earlier diagnosis and more aggressive and prolonged immunotherapy at the beginning could have had a more beneficial, long-term effect and might have ameliorated irreversible cerebellar destruction.

Regarding the similar clinical picture of the anti-DAGLA-CSF-positive patients and their severe clinical course, the detection of anti-DAGLA autoantibodies in the serum of one healthy control and in sera of patients with different clinical phenotypes challenged the specificity of anti-DAGLA autoantibodies as a cause of cerebellitis. However, our data indicate the existence of at least two subtypes of anti-DAGLA autoantibodies targeting distinct epitopes. Anti-DAGLA autoantibodies present in CSF samples of our index patients recognize a conformational epitope between amino acid 1 and 157, whereas the healthy control serum positive for anti-DAGLA in RC-IIFA and 16/17 sera of the DC recognize a linear epitope between amino acid 583 and 1042. Furthermore, cases of anti-DAGLA-positive cerebellitis were distinguishable from other clinical conditions by CSF analysis. High titers of anti-DAGLA antibodies (IgG titre >1:100) with positive tissue IIFA were only detected in CSF samples of patients with cerebellitis (P1-4 and DC9) and were intrathetically synthesised based on the calculated AI (P1-4), whereas the available CSF samples of patients with different clinical phenotypes (DC14: sensory neuropathy, DC15: epilepsy) were anti-DAGLA-negative (DC13) or only weakly positive (DC14, 1:1) and tissue-IIFA-negative.

The shortest DAGLA variant which is recognised by the patient CSFs still contains two short intracellular amino acid sequence sections (amino acid 1–22 and 82–101) in addition to two extracellular sequence stretches (amino acid 44–60 and 123–136). Therefore, it cannot be excluded that the patient anti-DAGLA autoantibodies bind to an intracellular, conformational epitope. As the strongest anti-DAGLA staining was observed in cerebellum, we would recommend to investigate epitope binding and functional effects of anti-DAGLA autoantibodies with living cerebellar neurons.
Although patients with autoantibodies against encephalitis-associated cell surface antigens like NMDA receptor respond well to immunosuppressive treatment with around 80% of patients improving after immunotherapy, treatment outcome in patients with autoantibodies directed against Purkinje cell antigens is significantly poorer, even when extracellular epitopes are recognised. An example are anti-Tr/DNER autoantibodies, which target an extracellular domain of the Purkinje-cell-expressed Delta and Notch-like epidermal growth factor-related receptor (DNER). Patients with these autoantibodies frequently suffer from Hodgkin lymphoma and develop rapidly progressive cerebellar ataxia. Despite successful tumour treatment significant neurological improvements were only observed in 41% or 50% of the patients. However, as a favourable outcome could be observed in some of the patients, especially in those with less decrease of cerebellar grey matter volumes, it can be suggested that early detection and aggressive treatment might improve prognosis. Similarly, only 40% of patients with antibodies against the Purkinje cell membrane protein mGLUR1 showed significant clinical improvement after immunotherapy, while severe cerebellar dysfunctions persisted in the rest of them. Again, delayed diagnosis and treatment with resulting irreversible neuronal damage were discussed as reasons for this poorer outcome.

In our epitope characterisation assays, only one (DC9) of 17 sera of the disease control group showed a similar clinical
phenotype and reactivity to that of the patient samples, indicating that cerebellitis-associated anti-DAGLA autoantibodies recognising conformational epitopes are less prevalent than anti-DAGLA autoantibodies targeting intracellular linear epitopes. In the clinical routine it might become challenging to differentiate these two groups of anti-DAGLA autoantibodies. Therefore, we recommend for the diagnostic workup, to consider only anti-DAGLA autoantibodies detected in tissue-IIF-positive CSF samples as a marker for a new form of progressive cerebellitis, particularly, in combination with CSF pleocytosis and signs of intrathecal autoantibody synthesis. Screening for anti-DAGLA autoantibodies should be performed using RC-IIF with DAGLA full length protein. Positive CSF samples should be confirmed by RC-IIF with the DAGLA 1–582 variant.

The limitation of our study is the small number of patients, which does not allow to estimate the incidence of the disease. The first descriptions of anti-NMDA receptor encephalitis with an estimated incidence of 1.5 per million population per year included only four53 and twelve54 patients. As there is a lack of larger patient numbers and confirmatory transfer animal studies, a causal antibody-disease relation cannot be proven. The clinical disease course as well as its paraclinical features are similar in all cases. The strong cerebellar inflammatory response in combination with an intrathecal antibody production of the anti-DAGLA antibodies might indicate a causal relationship. A further limitation is the small number of available CSF samples from index patients (n=4) and disease controls (n=5) with anti-DAGLA autoantibodies present in serum and future studies with well-characterised patient cohorts with paired serum/CSF samples need to confirm the finding that anti-DAGLA autoantibodies detected in cerebellitis patients recognise conformational epitopes. The identification of anti-DAGLA-associated cerebellitis seems important for several reasons. Without the autoantibody detection, an infectious cause of the disease might initially be assumed, which might delay immunosuppressive treatment. A rapid diagnosis and early, aggressive and prolonged immunotherapy might help to prevent severe neurological sequelae, as the disease progresses rapidly, and neuronal cell loss cannot be restored.

REFERENCES


Powell DR, Gay JP, Miljanowski N, et al. Diacylglycerol Lipase alpha knockout mice demonstrate metabolic and behavioral phenotypes similar to those of Cannabinoid receptor 1 knockout mice. Front Endocrinol (Lausanne) 2015;6:86.


