Quantified Csf Antibody Reactivity in Multiple Sclerosis: Specificity and Utility in Diagnosis

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Abstract

Introduction: Synthesis of clonal IgG is a consistent feature of patients with multiple sclerosis (MS). Whether oligoclonal bands (OCBs) represent unspecific disease bystanders or active components in MS pathology is an open question. The aim of this study was to develop further method to quantify and compare of the reactivity of cerebrospinal fluid (CSF) antibodies from patients with and without MS to evaluate this reactivity as a diagnostic tool box.

Methods: We collected CSF from totally 203 patients from three different cohorts with 100 clinically confirmed MS patients, 75 with other neurological diseases and 28 with clinical isolated symptom (CIS). Reactivity of cerebrospinal fluid (CSF) against purified myelin particles and biont anchored liposomes in a custom designed highly sensitive electrochemiluminescence (ECL)-based assay system. The diagnostic value of ECL score was assessed with receiver-operating-characteristic (ROC) curves.

Results: CSF from patients with clinically confirmed MS have higher reactivity towards purified myelin particles as compared with other neurological diseases (OND) with OCBs (p<0.01). The reactivity of antibodies against myelin correlated with their ability to induce complement-mediated cell death of oligodendrocytes (p<0.01). Using liposomes with defined lipid compositions we developed a tool box to quantify the CSF antibody reactivity directed against myelin in MS. The quantified reactivity against myelin demonstrated favorable accuracy as a biomarker in diagnosis of MS and differentiating MS from non MS OCB+ patients.

Keywords: Multiple sclerosis; Liposomes; Oligoclonal bands; Antibodies; Cerebrosides; Myelin

Abbreviations: Cer: Cerebrosides; CerS2: Ceramide synthase 2; Chol: Cholesterol; CIS: Clinically isolated syndrome; CNPase: 2',3'-Cyclic-nucleotide 3'-phosphodiesterase; CNS: Central nervous system; CSF: Cerebrospinal fluid; ECL: Electrochemiluminescence–based; ELISA: Enzyme-linked immunosorbent assay; FACS: Fluorescence-activated cell sorting; GFAP: Glial fibrillary acidic protein; Ig: Immunoglobulin; MAG: Myelin-associated glycoprotein; MBP: Membrane basic protein; MOG: Myelin oligodendrocyte glycoprotein; MS: Multiple sclerosis; OCBs: Oligoclonal bands; OND: Other neurological diseases; PC: Phosphatidylincholine; PLP: Myelin proteolipid protein; Ru(bpy)3, Ruthenium (II) triis-bipyridine-(4-methylsulfone)

Introduction

Multiple Sclerosis (MS) is a heterogeneous disease characterized by multifocal immune-mediated demyelinating lesions disseminated in time and space within the central nervous system (CNS) [1]. Autoimmune demyelination is complex and driven by different cells and components of the immune system [1]. One of the most consistent indications of an abnormal humoral response is the synthesis of clonal IgG in the cerebrospinal fluid (CSF) [2,3] routinely detected by isoelectric focusing and immunoblotting. Oligoclonal bands (OCBs) have been employed as a helpful test to assist multiple sclerosis diagnosis [4], however, OCBs present also in 2-18% of other neurologic disease [5]. Determination of the specificity of OCBs has become the focus of research in the recent years. Binding of myelin-specific antibodies from clonally expanded plasma cells in the CSF was observed against brain tissue in some MS patients [6]. Lipid reactivity of autoantibody in MS patients has been increasingly reported [7-9]. Also, the reactivity has been reported to be associated with pathogenesis and disease process of multiple sclerosis [10-14]. Recently the utilization of CSF or Serum autoantibody reactivity as a biomarker was increasingly noticed [15,16]. Although it has been recognized the antibody response towards a certain lipid or the combination of multiple lipids could be applied as biomarker, accurate assay and clinical large scale analysis haven't been done yet [7,14].

Methods

Patients: CSF samples were collected from patients at the University Medical Centre Götttingen (UMG), Germany and the Department of

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Received April 02, 2015; Accepted May 07, 2015; Published May 12, 2015


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Neurology, University Hospital Basel (UHB), Switzerland. The first cohort of patients from UMG included 29 OCB-positive patients with MS, 26 OCB-positive patients with other neurological diseases (OND OCB‘) and 22 OCB-negative patients with other neurological diseases (OND OCB).

The second cohort of subjects from the UHB included 43 OCB-positive MS patients, 28 OCB-positive patients with a clinically isolated syndrome (CIS) and 27 OCB-positive patients with other neurological diseases (OND OCB‘).

The third cohort of subjects from UMG included 28 OCB-positive patients with MS.

MS was diagnosed according to the 2010 McDonald criteria [17] and CIS patients had to present with at least two or more lesions on magnetic resonance imaging to be included [18]. The study was approved by the local ethics committees and informed consent from all patients participating in the investigation was obtained.

Antibodies and other reagents: The following primary antibodies were used: mouse anti-α-actin and mouse anti-CNPutide (2.3-Cyclonucleotide 3-phosphodiesterase) (Sigma-Aldrich); mouse anti-MAG (myelin-associated glycoprotein), mouse anti-MOG (myelin oligodendrocyte glycoprotein) (Millipore); mouse anti-MBP (membrane basic protein) (Sternberger); mouse anti-PLP (myelin proteolipid protein) (AA3); mouse anti-GFAP (Glia fibrillary acidic protein) (Santa Cruz Biotechnology); mouse anti-Neurofilament (Biocytex); secondary antibodies were purchased from Dianova and Invitrogen. Ceramide synthase (CerS2) knockout myelin (PC=phosphatidylcholine, Cer=brain cerebrosides; Chol=cholesterol). 1% biotin was supplemented to all of the lipid reagents. After speed-vacuum drying for 45 min at 30°C, the lipids were resuspended in HEPES buffer (100 mM NaCl, 50 mM HEPES pH 7.4).

Preparation of myelin and non-myelin membrane fractions

Animal care and use: All animal experiments were performed in accordance with laws for the use of animals in research under approval of the responsible local organization, Lebensmittel- und Veterinärinstitut Oldenburg. Wild type mice were housed under standard conditions and Ceramide synthase (CerS2) knockout mice were maintained on pathogen-free conditions with a mixed C57BL/6 × 129S4/SvJae background [19]. For preparation of myelin membrane, adult mice were anesthetized with CO2 and sacrificed by cervical dislocation followed by collection of the brains.

Myelin membrane was prepared as described previously [20]. Briefly, the brains from adult mice were homogenized in 0.32 M sucrose solution containing 5 mM EDTA, 10 mM HEPES pH 7.4. The samples were then applied to a two-step sucrose gradient (0.32 M and 0.85 M solution containing 5 mM EDTA, 10 mM HEPES pH 7.4). Subsequently, the suspensions were agitated at 40° C for 30 minutes and subjected to ten freeze-thaw cycles. Subsequently, the liposome extraction was done with a polycarbonate filter to obtain uniformly sized, unilamellar vesicles of approximately 100 nm in diameter.

Western blot analysis: Western blot analysis of membrane fractions was performed as described previously [24]. Briefly, mouse primary oligodendrocytes or myelin membrane fractions were mixed with lysis buffer (100 mM Tris, pH 7.5, 300 mM NaCl, 2 mM EDTA, and 2% Triton X-100 supplemented with Complete protease inhibitor cocktail) for 10 min on ice. After centrifugation, the protein concentration was measured using Bradford assay for the supernatant. A fraction of the samples was then mixed with sample buffer (20% glycerol, 4 mM EDTA, 4% SDS, 4% 2-mercaptoethanol, and 100 mM Tris-HCl, pH 6.8) and subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes using standard procedure. The proteins of interest were detected by enhanced chemiluminescence (Pierce/Thermo Scientific).

Electrochemiluminescence (ECL)-based assay system: The Meso Scale Discovery (MSD, Gaithersburg, MD, USA) assay platform utilizes Ruthenium (II) tris-bipyridine-4-(methylsulfone) (Ru(bpy)3) that, once conjugated to the analyte, serves as the tracer in competitive assays. The Ru(bpy)3-based tag (sulfo-tag) undergoes a rapid redox reaction that emits light in the presence of an applied voltage [25-26]. The assay was performed using high-bind plates (384-well) at room temperature. Briefly, 1 µg of membranes was added to each well for 1 hr followed by blocking with 25 µl of blocking solution composed of 3% BSA and 1% skim milk powder. As negative control, PBS buffer instead of membranes was applied. Subsequently, the plate was washed with washing buffer (50 mM Tris pH 7.5, 0.15 M NaCl, 0.02% Tween-20). 25 µl of CSF samples diluted in PBS (1:5) were added for 1 hr, followed by washing steps. Anti-human IgG sulfo-tag antibody was then added for 1 hr. After washing, the reading buffer was added and the ECL signal was measured with the SECTOR Imager 6000. The PBS ECL signal was subtracted from the membrane ECL signal to acquire the ECL score of membrane. All patient data was blinded during the analytical procedure.

ECL-based liposome assay: Liposome were immobilized onto plates through biotin-avidin binding as described by Matthews et al. [23]. In brief, 5 µl biotin anchored liposomes were immobilized on streptavidin coated plates (384-well) from the Meso Scale Discovery (MSD, Gaithersburg, MD, USA). After 1h incubation, the plates were washed three times with HEPES buffer (100 mM NaCl, 50 mM HEPES pH 7.4). Subsequently, 25 µl of 1:5 diluted CSF samples were added for 1 hr, followed by washing steps. The plates were then incubated with anti-human IgG sulfo-tag antibody for 1 hr. After washing, the reading buffer was added and the ECL signal was measured with the SECTOR Imager 6000. All patient data was blinded during the analytical procedure.
Cytotoxicity assay: Primary cultures of mouse oligodendrocytes were prepared as described previously [27]. Cells at day 4 in culture were incubated with 1% pig complement and CSF samples. The cell viability was assessed after 24 hr using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. All patient data was blinded during the analytical procedure.

Statistical analysis: To analyse the ECL-based data, two-way ANOVA was performed using GraphPad Prism 5 statistical software (GraphPad Software, La Jolla, CA). For cytotoxicity analysis, one-way ANOVA was performed using GraphPad Prism 5. Values are expressed as mean ± standard deviation of the mean (SD). To analyse the correlation between ECL score and cell viability, Pearson correlation test was performed. The ability of ECL score to help with identifying multiple sclerosis was evaluated by receiver-operating-characteristic (ROC) curves [28-29]. The analysis was conducted firstly among all the patients regardless of presence of OCBs, and subsequently performed only within patients with CSF OCBs. We calculated the area under the ROC curve (AUC) and its 95% confidence interval. Also, we compared the AUC value between autoantibody response and IgG index, which is applied as an additional support of diagnosis together with OCBs according to the latest McDonald criteria [30].

Results

Since there is a high degree of conservation of myelin components between mice and man, we used purified myelin from mice for our assays [31]. The purity of the membrane fractions was confirmed by Western blot analysis, antibodies against neuronal, astrocytic and myelin proteins (Figure S1).

To obtain a sensitive and quantitative detection system, we set up a custom designed ECL-based assay. Myelin particles were absorbed to carbon coated plates and the specificity of the assay system was evaluated with antibodies against myelin proteins and control antibodies followed by secondary sulfo-tag-coupled antibodies. A membrane fraction depleted of myelin was used as a reference (non-myelin fraction). Myelin protein antibodies showed higher reaction towards myelin than non-myelin (ratio=1), verifying the specificity of this method and serving at the same time as the positive control for further antibody detection (Figure S2-A). Multiple trials in which the fractions were placed at different plate locations were conducted at different time point. No inter-assay or intra-assay variability was observed.

To analyse the reactivity of IgG antibodies we used CSF from the first cohort (discovery samples cohort), which included 29 MS-patients, 26 OND OCB+ -patients and 22 OND OCB- -patients (Table 1 and Table S1). CSF from patients with MS showed higher reactivity towards myelin as compared with both groups with OND. Conversely, reactivities against the non-myelin membrane fraction did not differ significantly between MS and OND OCB+ patient (Figure 1A). In

<table>
<thead>
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<th>MS OCB+</th>
<th>CIS OCB+</th>
<th>OND OCB+</th>
<th>OND OCB-</th>
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<tr>
<td>Number of patients</td>
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<td>28</td>
<td>53</td>
<td>22</td>
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<td>Number of women (%)</td>
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<td>23(43.4%)</td>
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<td>56</td>
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<td>28~49</td>
<td>21~69</td>
<td>42~71</td>
<td>35~58</td>
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<tr>
<td>ECL score towards myelin (SD)</td>
<td>13602.5(12410.4)</td>
<td>9602.5(13510.4)</td>
<td>5645.1(7526.3)</td>
<td>2192.3(2238.3)</td>
</tr>
<tr>
<td>ECL score towards non-myelin (SD)</td>
<td>9220.1(10894.7)</td>
<td>6220.1(7498.7)</td>
<td>5083.4(10050.6)</td>
<td>1254.5(1075)</td>
</tr>
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<td>IgG concentration mg/L (SD)</td>
<td>38.9 (39.3)</td>
<td>40.3 (41.2)</td>
<td>45.6 (36.2)</td>
<td>18.2 (6.1)</td>
</tr>
<tr>
<td>IgG Index (SD)</td>
<td>0.96 (0.548)</td>
<td>0.87 (0.632)</td>
<td>0.91 (0.66)</td>
<td>0.47 (0.04)</td>
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Table 1: Clinical and demographic characteristics of recruited patients.
addition, there was no correlation between the ECL score against myelin and the level of intrathecal IgG in the CSF from MS patients, indicating the absolute amount of intrathecal IgG do not affect the CSF anti-myelin reaction in ECL-based assay (Figure S2-B).

In addition, when the reactivity of the CSF against myelin and non-myelin fractions was compared, MS patients showed a higher reactivity against the myelin components (p<0.05), whereas this was not observed in any of the OND groups (Figure 1A). Thus, CSF in patients with MS, but not OND has a significantly higher reactivity towards myelin as compared with the non-myelin membrane fraction.

To obtain evidence whether the CSF has pathogenic potential, we set up a complement-based lysis assay of primary oligodendrocytes in culture. Within a few days in culture oligodendrocytes differentiate into cells expressing all major known myelin proteins and lipids [32] (Figure S3). CSF and complement was applied to the cells in order to determine a possible antibody induced cytotoxic effect indicated by a reduction in cell number. As a control we used complement in the absence of CSF or complement together with CSF from patients lacking OCBs. Both treatments failed to induce significant cell death ruling out non-specific effects of the complement. However, when the CSF of a subgroup of MS patients with the highest reactivity towards myelin was selected (ECL score against myelin higher than mean), a significant reduction in cell number was observed (Figure 1B). The degree of cytotoxicity correlated with myelin reactivity in the ECL-based assay (Figure 1C). Thus, our study uncovers a subgroup of patients with MS and reactivity of CSF against myelin.

To validate our results, the ECL-based membrane assay analysis was performed with a second independent cohort of patients (validation cohort) with OCBs including 43 patients with MS, 28 with CIS and 27 with OND (Table 1, Table S1). Again, a higher reactivity of CSF from the patients with MS towards myelin as compared with OND was observed. This was not the case for the CIS patients. Moreover, when the reactivity of the CSF against myelin and non-myelin membrane fractions was compared, a higher reactivity against myelin was found for the MS patients but not for CIS/OND. In contrast, OND CSF samples exhibited higher reactivity against the non-membrane fraction as compared with myelin (Figure 2). Thus, we confirmed the difference of antibody reactivity between OCB+ MS and OCB+ OND patients in two separate cohorts.

To assess the antibodies response against the major myelin lipids, we prepared liposomes with defined lipid compositions. Liposomes were generated with only phosphatidylcholine (PC) or with phosphatidylcholine and brain cerebrosides (PC/Cer) containing long-chain galactosylcerebrosides (d16:1/24:0) as the predominant species. Phosphatidylcholine and brain cerebrosides (PC/Cer) containing long-

Purified myelin particles from wild-type and CerS2 deficient mice were absorbed to carbon coated plates and CSF reactivity was determined. There was a significantly higher reactivity towards wild-type as compared with CerS2 knockout myelin particles. To analyze whether these results were due to the differences in lipids, we extracted lipids from wild-type and CerS2 knockout myelin, generated liposomes and


Figure 2: Analysis of the validation cohort. The reactivity of CSF with OCBs from a second cohort of patients against myelin and non-myelin membranes was assessed using the ECL-based assay system. CSF from MS patients with OCB revealed higher reactivity against myelin as compared with ONDs with OCBs. When reactivity against myelin and non-myelin membrane was compared, CSF from these MS patients showed higher reactivity to myelin, while CSF from OND with OCB exhibited higher reactivity against the non-myelin fraction. CIS OCB did not show any difference in reactivity against myelin as compared with the non-myelin membrane fraction (*p < 0.05, **p < 0.01, ***p < 0.001; two-way ANOVA).

Figure 3: CSF reactivity against cerebrosides. (A) The CSF reactivity of 28 MS with OCBs patients was tested in an ECL-based assay against PC, PC/Cer and PC/Chol/Cer liposomes. The reaction against PC/Cer and PC/Chol/Cer liposomes was significantly higher as compared with PC liposomes (*p < 0.05, **p < 0.01, ***p < 0.001; one-way ANOVA). The CSF reactivity of 28 MS with OCBs patients against PC/Chol/Cer (B) and PC/Cer (C) liposomes correlated with the reactivity against myelin liposomes (Pearson correlation test). (D) The reactivity of CSF of 28 MS patients with OCBs patients against myelin particles from wild-type, CerS2 knockout mice myelin and non-myelin was assessed using the ECL-based assay system. CSF reactivity towards wild-type myelin particles was significantly higher as compared with CerS2 knockout myelin and non-myelin (*p < 0.05, one-way ANOVA). (E) Significantly higher reactivity towards wild-type as compared with CerS2 knockout myelin liposomes (**p < 0.01; one-way ANOVA).
immobilized the liposomes with a biotin linker to streptavidin coated plates. There was a higher reactivity towards liposomes prepared from wild-type as compared with CerS2 knockout myelin, demonstrating that antibodies against myelin are mainly directed towards cerebrosides (Figure 3D and 3E).

**ROC curve:** We analyzed the ability of ECL score in diagnosing multiple sclerosis and compared it with IgG Index with all the 208 patients in three cohorts. The AUC value of ECL score against myelin is 0.779 with 95% CI interval from 0.717 to 0.842. The AUC value of IgG index is 0.739 with 95% CI interval from 0.657 to 0.821 (Figure 6A and 6B). In patients with OCBs, the AUC value of ECL score is 0.744 with 95% CI of 0.671 to 0.817 whereas the AUC value of IgG Index in patients with positive OCBs is 0.638 with 95% CI of 0.535 to 0.740 (Figure 6C and 6D).

**Discussion**

In this study we firstly compared the qualitative differences of CSF antibody reactivities isolated from OCB+ patients with MS and other neurological diseases. We find that CSF from patients with MS has higher reactivity towards purified myelin particles as compared with control OND subjects with OCBs. Furthermore, by comparing myelin from wild-type and CerS2 knockout mice and by using liposomes with defined lipid compositions, we find that antibodies are primarily directed against cerebrosides. Finally, we assessed the ability of the autoantibody reactivity against myelin in diagnosing multiple sclerosis with samples from 208 patients. Comparing IgG index, which is an established method to support MS diagnosis, the AUC value of ECL score indicated its favorable potentiality to be applied as a biomarker for MS diagnosis. The cytotoxicity of CSF antibody was confirmed in cytotoxicity assay. Together, these results support the existence of anti-cerebrosides antibody in CSF of OCB+ MS patients, and revealed the potential utilization of autoantibody response as biomarker for multiple sclerosis.

Our study is in line with a recently published study demonstrating the pathogenic role of antibodies in the serum of patients with MS [10]. Using a myelinating co-culture system the authors demonstrate complement-mediated demyelination and axonal loss induced by serum-derived antibodies from MS patients. As in our study, a subset of MS patients had pathogenic antibodies as determined by the complement lysis assay. Interestingly, a recent study demonstrated high antibody titers against Kir4.1 in a group of MS patients [34]. It suggested that the reactivity towards self-antigens may allow the discrimination of MS subtypes characterized by an abnormal activation of the humoral immune system. The sensitive assay system established in this study will be useful to screen for antibodies reacting against myelin. In a previous study using flow cytometry enhanced level of anti-myelin antibodies have been detected in the serum of MS patients [35]. Advantages of our platform as compared with traditional enzyme-linked immunosorbent assay (ELISA) or fluorescence-activated cell sorting (FACS) for performing sandwich immunoassays include high sensitivity (within the sub-picogram range), high capture capacity and a dynamic range of 3-4 log.

The evidence of the key role of local antibodies production in MS pathogenic process is increasing [11]. CSF derived autoantibodies against myelin proteins and lipids have been described previously in MS patients [2,7,9,36-38]. Evidence has recently been presented that some antibodies against sulfatides only recognize the lipid when it is in a complex with other lipids and in its natural environment [9]. Here, we used purified myelin particles isolated without denaturing agents in order to preserve the natural membrane environment of myelin protein and lipids. In most previous studies, lipids were directly spotted onto membranes or plates for their detection by antibodies from serum or
CSF [8]. These studies led to the detection of lipid-specific antibodies against galactosylceramide, sulfatide, sphingomyelin and oxidized lipids [7,9,36-38]. Since formation of conformation dependent epitopes might be dependent on membrane insertion, we used bilayered liposomes as a method to prepare artificial membranes with a defined lipid composition. This allowed us to identify cerebroside specific antibody reaction in a subpopulation of MS patient. There was a strong correlation between the reactivity of CSF towards myelin particles and liposomes containing cerebrosides suggesting that antibody responses against myelin are primarily directed against cerebrosides.

Lipid(s) and the autoantibody response towards lipid(s) in serum and CSF have been increasingly investigated as biomarkers [4,14-15], our study is unique that we are able to isolate purified myelin particle without detergent and immobilize the particle with a highly sensitive assay. The platform developed in our study allows detection of combination of different lipids using synthesized liposomes. AUC value of ECL score is higher than IgG index in all the patients and patients with positive OCBs, indicating ECL myelin score is a promising biomarker to be used alone or as additional analysis to help differentiating MS with non MS in OCB positive patients. These results of our study encouraging further investigation of the clinical use of antibody response in multiple sclerosis.

Conclusion

The ECL-based system together with the complement-mediated cell lysis assay expands the currently available toolbox for the detection of pathogenic antibodies in MS and related diseases. The quantified autoantibody response with the method described in our study might be applied as biomarkers assisting diagnosis of multiple sclerosis.

Acknowledgements

The work was supported by an grants from the German Research Foundation (SI 746/9-1; TRR43; SFB 645; B2 to KW) and the Klaus Tschira-Stiftung. Xingwen Sun and Mikael Simons had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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