ANNALS of Clinical and Translational Neurology
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RESEARCH ARTICLE

Quantified CSF antibody reactivity against myelin in multiple sclerosis

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Funding Information
The work was supported by grants from the German Research Foundation (SI 746/9-1; TRR43; SFB 645, B2 to KW) and the Klaus Tschira-Stiftung.

Received: 5 July 2015; Revised: 21 September 2015; Accepted: 12 October 2015

Annals of Clinical and Translational Neurology **2015; 2(12): 1116–1123**

doi: 10.1002/acn3.264

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Abstract

Background: 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 a method to quantify and compare the reactivity of cerebrospinal fluid (CSF) antibodies from patients with and without MS. **Methods**: We collected CSF from 262 patients from two different cohorts, which included 148 patients with MS and 114 with other neurological diseases (OND). We established a highly sensitive electrochemiluminescence (ECL)-based assay to measure CSF antibody reactivity against purified myelin particles and biotin anchored liposomes. The diagnostic value of the ECL score against myelin particles was assessed with receiver operating characteristic curves. **Results**: CSF from patients with MS have higher reactivity toward purified myelin particles as compared to those with OND with OCBs. Using liposomes with defined lipid compositions and myelin particles from ceramide synthase 2 (*CerS2*) knockout mice, we find that some of the CSF antibody reactivity is directed against cerebrosides. **Conclusion**: The ECL-based assay system expands the currently available toolbox for the detection of autoantibodies in MS and related diseases.

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.[1] Autoimmune demyelination is complex and driven by different cell types 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. However, oligoclonal bands (OCBs) are present also in a significant portion of other neurologic diseases.[4] One problem has been the difficulty in defining their specific target antigens. For example, recombinant antibodies prepared from clonally expanded plasma cells and B cells from patients with MS failed to react against brain tissue or myelin proteins.[5] In addition, CSF immunoglobulins from patients with MS did not show reactivity against cultured rat and human oligodendrocytes.[6] In contrast,

binding of myelin-specific antibodies from clonally expanded plasma cells in the CSF was observed against brain tissue in some MS patients.[7,8] Moreover, nonmyelin antigens such as Epstein–Barr virus proteins have been shown as a target of antibodies in MS.[9] Lipid reactivity of autoantibodies has also been reported and shown to be associated with disease progression of MS.[10–17,18] Recently, the utilization of CSF or serum autoantibody reactivity as a biomarker was increasingly noticed.[11,19–21] Although it has been recognized that the antibody response toward a certain lipid or the combination of lipids could be applied as biomarker, accurate assays, and large-scale clinical analysis are lacking.[17,22] The aim of our study was to develop an assay system that discriminates CSF antibody reactivity between MS and non-MS patients with OCBs. We developed an electrochemiluminescence (ECL)-based assay system that uses CSF from patients as a source for capture antibodies for the detection of myelin particles isolated from mice or reconstituted as liposomes.

Methods

Patients

CSF samples were collected from patients at the University Medical Centre Göttingen (UMG), Germany and the Department of Neurology, University Hospital Basel (UHB), Switzerland. The first cohort of patients from UMG included 77 OCB-positive patients with MS (MS OCB^+), 51 OCB-positive patients with other neurological diseases (OND OCB^+), and 36 OCB-negative patients with other neurological diseases (OND OCB^-) (Table S1).The second cohort of subjects from the UHB included 71 OCB-positive multiple sclerosis/clinically isolated syndrome (MS/CIS) patients, and 27 OCB-positive patients with other neurological diseases (OND OCB^+) (Table S1). We only included patients with OCBs present in only CSF, but not the serum sample into the study (OCB type 2 and type 3 pattern according to Freedman et al.[23]). MS was diagnosed according to the 2010 McDonald criteria.[23] The study was approved by the local ethics committees and informed consent from all patients participating in the investigation was obtained.

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, Germany. 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.[24] For preparation of myelin membrane, adult mice were anesthetized with CO_2 and killed by cervical dislocation followed by collection of the brains.

Myelin membrane was prepared as described previously.[25] Briefly, the brains from adult mice were homogenized in 0.32 mol/L sucrose solution containing 5 mmol/L ethylene diamine tetraacetic acid (EDTA), 10 mmol/L N′-2 Hydroxyethylpiperazine-N′-2 ethanesulphonic acid (HEPES) pH 7.4. The samples were then applied to a two-step sucrose gradient (0.32 and 0.85 mol/L sucrose) and were centrifuged at 74,000*g* for 30 min. The interfaces (crude myelin) were diluted with water and centrifuged at 74,000*g* for 15 min. The pellets were washed three times with water and centrifugation at 12,500*g* for 10 min, and the procedure was repeated once more to obtain purified myelin. The pellets from the first gradient step were washed and applied to two more gradient steps to obtain nonmyelin membrane fraction.

Preparation of liposomes

Liposomes were prepared as described previously.[26,27] Briefly, five lipid reagent solutions were prepared: PC, PC + 15% Cer, PC + 34% Chol + 15% Cer, wild-type myelin, and *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 mmol/L NaCl, 50 mmol/L HEPES pH 7.4). The suspensions were agitated at 40°C for 30 min and subjected to 10 freeze-thaw cycles. Subsequently, the liposome extrusion was done with a polycarbonate filter to obtain uniformly sized, unilamellar vesicles of ~100 nm in diameter.

ECL-based assay system

The Meso Scale Discovery (MSD, Gaithersburg, MD) assay platform utilizes Ruthenium (II) tris-bipyridine-(4-methylsulfone) [Ru(bpy)3] for detection. The Ru(bpy)3-based tag (sulfo-tag) undergoes a rapid redox reaction that emits light in the presence of an applied voltage.[28,29] 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 h followed by blocking with 25 μL of blocking solution composed of 3% Bovine serum albumin (BSA) and 1% skim milk powder. As negative control, Phosphate-buffered saline (PBS) buffer instead of membranes was applied. Subsequently, the plate was washed with washing buffer (50 mmol/L Tris pH 7.5, 0.15 mol/L NaCl, 0.02% Tween-20). 25 μL of CSF samples diluted in PBS (1:5) were added for 1 h, followed by washing steps.

Anti-human IgG sulfo-tag antibody was then added for 1 h. After washing, the reading buffer was added and the ECL signal was measured with the SECTOR Imager 6000 Meso Scale Diagnostics , Rockville, MD, USA. The PBS ECL signal was subtracted from the membrane ECL signal to acquire the ECL score of membrane. Experiments were performed with the operator being blinded for the patients′ diagnosis.

ECL-based liposome assay

Liposome were immobilized onto plates through biotin–avidin binding as described by Smith et al.[27] In brief, 5 μL biotin anchored liposomes were immobilized on streptavidin-coated plates (384-well) from MSD, Gaithersburg, MD. After 1 h incubation, the plates were washed three times with HEPES buffer (100 mmol/L NaCl, 50 mmol/L HEPES pH 7.4). Subsequently, 25 μL of 1:5 diluted CSF samples were added for 1 h, followed by washing steps. The plates were then incubated with anti-human IgG sulfo-tag antibody for 1 h. After washing, the reading buffer was added and the ECL signal was measured with the SECTOR Imager 6000. Experiments were performed with the operator being blinded for the patients′ diagnosis.

Statistical analysis

To analyze the ECL-based data, two-way analysis of variance (ANOVA) was performed using IBM SPSS 20 statistical software, Armonk, NY, USA. For cytotoxicity analysis, one-way ANOVA was performed. Values are expressed as mean ± standard deviation of the mean (SD). Univariate analysis was performed to evaluate the association of CSF total protein concentration, serum albumin concentration, CSF albumin concentration, albumin CSF/serum ratio, CSF IgG concentration, serum IgG concentration, IgG Index, ECL antimyelin score, and confirmed diagnosis of MS. Multivariable logistic regression was conducted including variables with significant association with confirmed MS diagnosis in univariate analysis. The ability of ECL score to help with identifying MS was evaluated by receiver operating characteristic (ROC) curves.[30,31] 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. We compared the AUC value ECL antimyelin score and IgG index, which was recommended as an supportive marker for MS diagnosis by the 2010 McDonald criteria.[23] Logistic regression and area under the ROC curve are two standard methods evaluating biomarkers.[32–35] Logistic regression analyzes the association of the value of biomarker with binary diagnosis. The ROC curve plots the sensitivity of all possible values of the threshold against one minus the specificity. The ROC curve allows the visualization of the ability of a biomarker to identify the disease state. The AUC value is an informative metric of the accuracy of predictions. An AUC value close to 1 indicates an excellent test whereas a low AUC value (near 0.5) represents a low accuracy of the test. AUC value is especially useful in comparing different tests.

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.[36] The purity of the membrane fractions was confirmed by western blot analysis, antibodies against neuronal, astrocytic, and myelin proteins (Methods see Data S1, Fig. 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 (nonmyelin fraction). Myelin protein antibodies showed higher reaction toward myelin than nonmyelin (ratio > 1), verifying the specificity of this method and serving at the same time as the positive control for further antibody detection (Fig. S2). Multiple trials in which the fractions were placed at different plate locations were conducted at different time point. No significant intra-assay variability was observed. Based on five repeated trials, the interassay variability ranged from 2% to 5% (SD/Mean × 100) demonstrating the favorable reproducibility of the assay (Fig. S2A).

To analyze the reactivity of IgG antibodies, we used CSF from the first cohort (discovery samples cohort), which included 43 MS patients, 51 OND OCB^+ patients, and 36 OND OCB^- patients (Tables S1 and S2). CSF from patients with MS showed higher reactivity toward myelin as compared with OND. Conversely, reactivities against the nonmyelin membrane fraction did not differ significantly between MS and OND OCB^+ patient (Fig. 1A). In addition, there was no correlation between the ECL score against myelin and the level of intrathecal IgG in the CSF from MS patients, indicating that the absolute amount of intrathecal IgG does not affect the CSF antimyelin reaction in the ECL-based assay (Fig. S2). Thus, CSF in patients with MS, but not OND has a significantly higher reactivity toward myelin as compared with the nonmyelin membrane fraction.

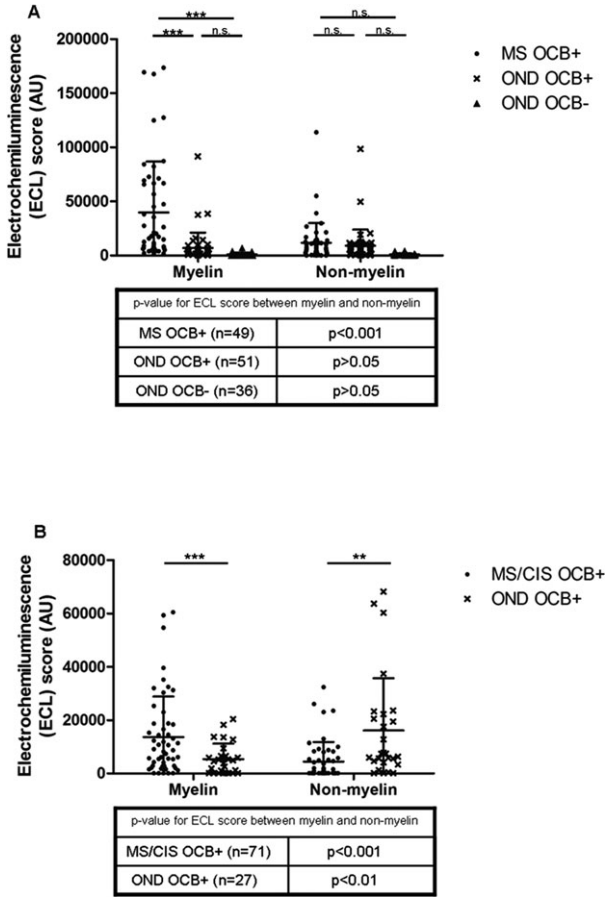

Figure 1. ECL-based assay analysis of first discovery cohort. (A) CSF from MS patients with OCB^+ revealed higher reactivity against myelin as compared with OND with (OCB^+) and without OCB (OCB^-). When reactivity against myelin and nonmyelin membrane was compared, CSF from MS patients showed higher reactivity to myelin. OND OCB^+ and OND OCB^- did not show any difference in reactivity against myelin as compared with the nonmyelin fraction (***$P < 0.001$; two-way ANOVA). (B) The reactivity of CSF with OCBs from a second cohort of patients against myelin and nonmyelin membranes was assessed using the ECL-based assay system. CSF from MS/CIS patients with OCB revealed higher reactivity against myelin as compared with OND OCB^+ ($P < 0.001$). When reactivity against myelin and nonmyelin membrane was compared, CSF from these MS/CIS patients showed higher reactivity to myelin ($P < 0.001$), while CSF from OND with OCB exhibited higher reactivity against the nonmyelin fraction ($P < 0.01$). **$P < 0.01$, ***$P < 0.001$; two-way ANOVA. ECL, electrochemiluminescence; CSF, cerebrospinal fluid; MS, multiple sclerosis; OCB^+, oligoclonal bands positive; OND, other neurological diseases; ANOVA, analysis of variance.

To validate our results, the ECL-based membrane assay analysis was performed with a second independent cohort of patients (validation cohort) with OCBs including 71 patients with MS (including 28 patients with CIS), and 27 with OND (Tables S1 and S2). Again, a higher reactivity of CSF from the patients with MS toward myelin as compared with OND was observed. Moreover, when the reactivity of the CSF against myelin and nonmyelin membrane fractions was compared, a higher reactivity against myelin was found for the MS patients, but not for OND. In contrast, OND CSF samples exhibited higher reactivity against the nonmyelin fraction as compared with myelin (Fig. 1B). Thus, we confirmed the difference in 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 PC or with PC/Cer containing long-chain galactosylcerebrosides (d16:1/24:0) as the predominant species. Since cholesterol is known to modulate glycolipid conformation, cholesterol was included into another set of liposomes (PC/Cer/Chol). Using mouse anti-GalC IgG as primary antibody as positive control and anti-MOG (myelin-oligodendrocyte glycoprotein) antibody serving as a negative control, we validated the ECL-based liposome assay in five repeated measurements (Fig. S2C). We used CSF from 43 MS/CIS OCB^+ patients, 25 OND OCB^+ patients, and 14 control OCB^- patients from the first cohort (Table S1 and S3), and found that its reactivity was significantly higher toward liposomes containing PC/Cer and PC/Chol/Cer as compared with PC. No significant differences were observed between PC/Cer and PC/Chol/Cer containing liposomes (Fig. 2A). In addition, there was a strong correlation between the reactivity of CSF toward myelin particles and both PC/Cer and PC/Chol/Cer containing liposomes suggesting that antibody responses against myelin are primarily directed against cerebrosides (Fig. 2B and C). To test this, we prepared myelin particles from wild-type and *CerS2* knockout mice generated from *CerS2* gene trap embryonic stem cells. *CerS2* deficient mice form myelin, but have strongly reduced levels of very long-chain fatty acid containing galactosylceramide/sulfatides.[37] 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 toward wild type as compared with *CerS2* knockout myelin particles (Fig. 3A). To analyze whether these results were due to the differences in lipids, we extracted lipids (Methods see Data S1) from wild-type and *CerS2* knockout myelin, generated liposomes and immobilized the liposomes with a biotin linker to streptavidin-coated plates. There was a higher reactivity toward liposomes prepared from wild type as compared with *CerS2* knockout myelin, demonstrating that some of the antibodies against myelin are directed toward cerebrosides (Fig. 3B).

Using ROC curve, we analyzed the ability of the ECL score against myelin particle in diagnosing MS and compared it with the IgG Index in all 262 patients. If ROC

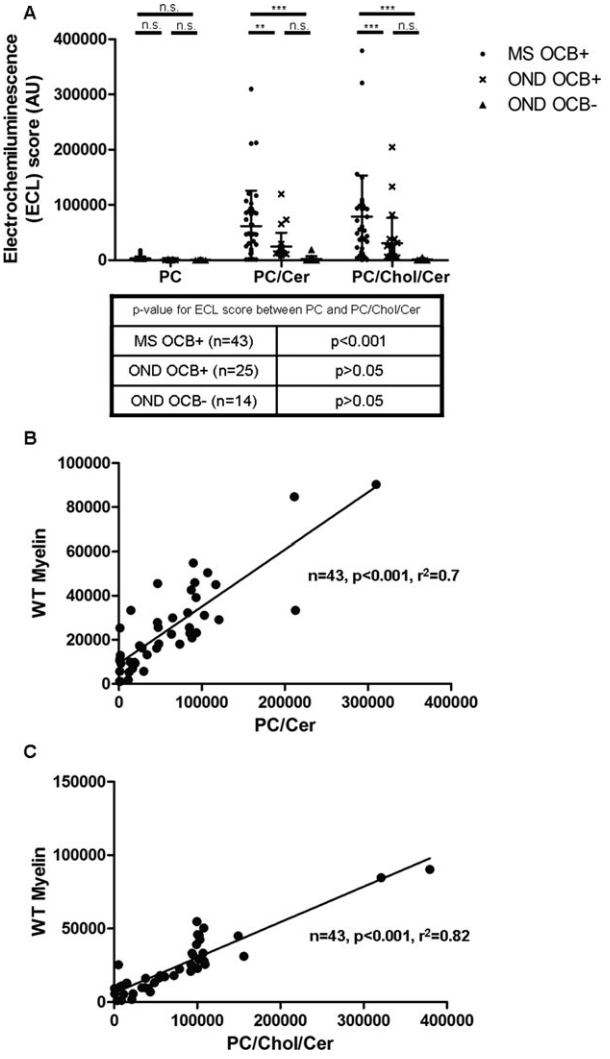

Figure 2. CSF reactivity against liposomes containing cerebrosides. (A) ECL-based liposome assay using PC, PC/Chol/Cer and PC/Cer liposomes. The reaction against PC/Cer ($P < 0.01$) and PC/Chol/Cer ($P < 0.001$) liposomes of MS OCB$^+$ CSF was significantly higher as compared with OND OCB$^+$ CSF, and OND OCB- CSF ($P < 0.001$, $P < 0.001$). MS OCB$^+$ CSF showed higher reaction against PC/Chol/Cer than PC ($P < 0.01$). (**$P < 0.01$, ***$P < 0.001$; two-way ANOVA). (B and C) The CSF reactivity of MS OCB$^+$ patients against PC/Cer (B) and PC/Chol/Cer (C) liposomes correlated with the reactivity against myelin liposomes (Pearson correlation test). CSF, cerebrospinal fluid; ECL, electrochemiluminescence; PC, phosphatidylcholine; Chol, cholesterol; Cer, brain cerebrosides; MS, multiple sclerosis; OCB, oligoclonal bands; OND, other neurological diseases; ANOVA, analysis of variance.

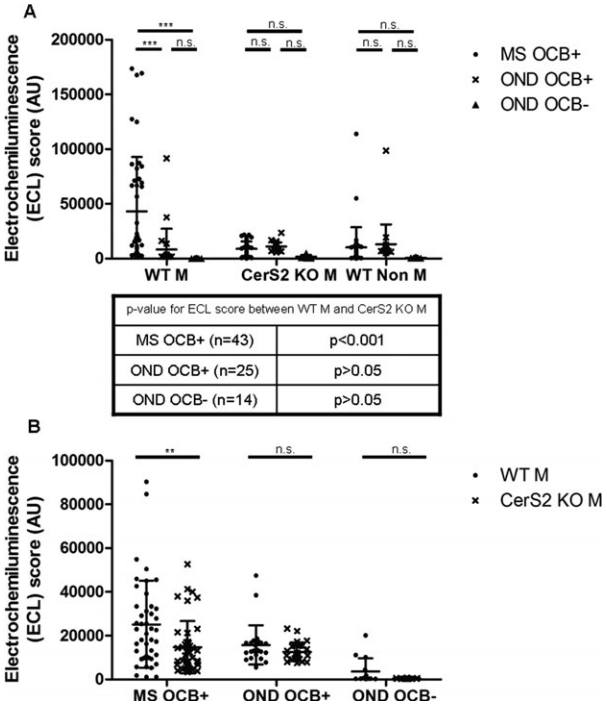

Figure 3. CSF reactivity against myelin particles and liposomes from CerS2 KO mice. (A) The CSF reactivity of 43 MS OCB$^+$, 25 OND OCB$^+$, and 14 OND OCB patients was tested in an ECL-based membrane assay against wild-type myelin particle (WT M), *CerS2* KO mice myelin particle (CerS2 KO M), and nonmyelin particle (WT Non-M). The antibody reactivity of MS OCB$^+$ patients against WT M reactivity was significantly higher than that of OND OCB$^+$ patients ($P < 0.001$), and control OCB$^-$ patients ($P < 0.001$). The CSF of MS OCB$^+$ patients showed stronger reactivity toward WT M than toward CerS2 KO M ($P < 0.001$). (B) The CSF reactivity of 43 MS OCB$^+$, 25 OND OCB$^+$, and 14 OND OCB$^-$ patients was tested in an ECL-based liposome assay against wild-type myelin liposome (WT M), and *CerS2* KO mice myelin liposome (CerS2 KO M). The CSF reactivity of MS OCB$^+$ patients against WT M was significantly higher than toward CerS2 KO M ($P < 0.01$). The CSF reactivity of OND OCB$^+$ and OCB$^-$ patients against WT M and CerS2 KO M were not significantly different ($P > 0.05$). (**$P < 0.01$, ***$P < 0.001$) CSF, cerebrospinal fluid; KO, knockout; MS, multiple sclerosis; OCB, oligoclonal bands; OND, other neurological diseases; ECL, electrochemiluminescence.

analysis is conducted in all patients, including patients with OCBs and without OCBs, the AUC value of ECL score against myelin particles in differentiating MS from non-MS is 0.799 with a 95% CI interval from 0.740 to 0.857. Comparing with the IgG index, which is an established method to detect intrathecal antibody synthesis, no significant difference of AUC is observed (AUC = 0.774, 95% CI 0.711–0.837, $P = 0.28$) (Fig. 4A and B), indicating a comparable ability of ECL myelin score with the IgG index in diagnosing MS. If ROC analysis is conducted only in patients with OCBs, the AUC value of ECL score is 0.753 with 95% CI of 0.685–0.822 whereas the AUC value of IgG Index is 0.686 with 95% CI of 0.604–0.767 comparing MS with non-MS. When comparing with the IgG index, the ECL myelin score showed a tendency to have better performance ($P = 0.065$) in differentiating MS from non-MS in OCB-positive patients.

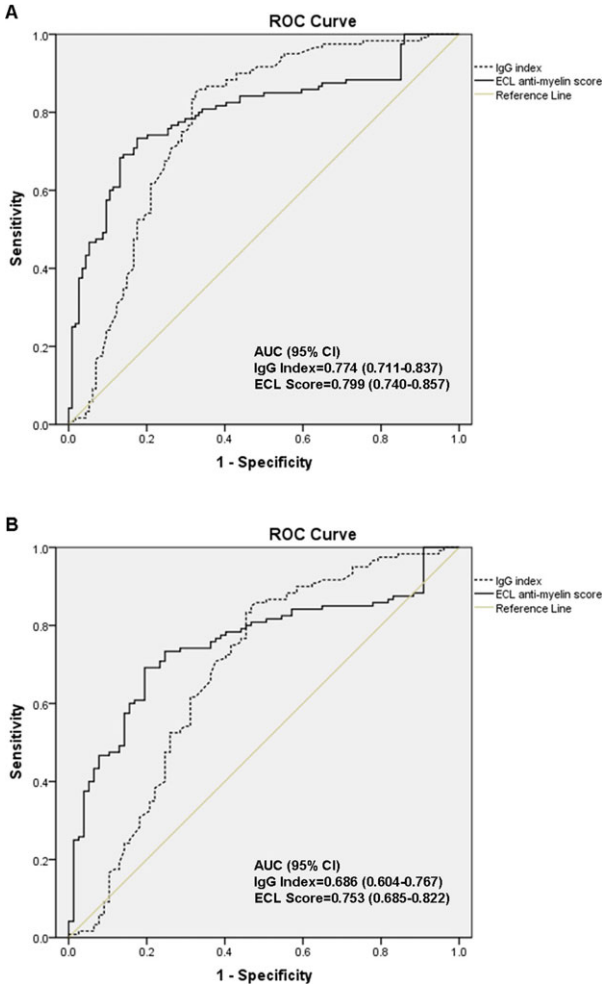

Figure 4. ROC curves and AUC value of ECL myelin score and IgG Index in all the patients (A) and in OCB-positive patients (B) in differentiating MS from non-MS. ROC, receiver-operating-characteristic; AUC, area under the curve; ECL, electrochemiluminescence; OCB, oligoclonal bands; MS, multiple sclerosis.

Discussion

In this study, we firstly compared the qualitative differences in CSF antibody reactivities isolated from OCB^+ patients with MS and OND. We found that CSF from patients with MS had higher reactivity toward 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 found that antibodies were primarily directed against cerebrosides. Together, these results support the existence of anti-cerebrosides antibodies in the CSF of OCB^+ MS patients, and revealed the potential utilization of an autoantibody response against myelin as a biomarker for MS.

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.[38] 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.

CSF-derived autoantibodies against myelin proteins and lipids have been described previously in MS patients.[2,8,10,20,21,39–41] 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.[10] 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.[11] These studies led to the detection of lipid-specific antibodies against galactosylceramide, sulfatide, sphingomyelin, and oxidized lipids.[39,40] 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 indentify cerebroside-specific antibody reaction in a subpopulation of MS patient. There was a strong correlation between the reactivity of CSF toward myelin particles and liposomes containing cerebrosides suggesting that antibody responses against myelin were primarily directed against cerebrosides.

Lipid(s) and the autoantibody response toward lipid(s) in serum and CSF have been increasingly investigated as biomarkers.[3,11,17] Our study is unique that we were able to isolate purified myelin particle without detergent and immobilize the particle in a highly sensitive assay. The platform developed in our study allows detection of a combination of different lipids using synthesized liposomes. The high AUC value of the ECL myelin score indicated its potential as a biomarker for MS. These results of our study encourage further investigation of the clinical use of antibody responses using the ECL-based assay system in MS and related diseases.

Acknowledgments

The work was supported by grants from the German Research Foundation (SI 746/9-1; TRR43; SFB 645, B2 to K. W.) and the Klaus Tschira-Stiftung.

Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Purity control of myelin, nonmyelin membrane, and oligodendrocytes. (A) Myelin and nonmyelin membrane fractions were prepared from adult mice brain density gradient centrifugation. Western blot analysis was performed using antibodies against indicated proteins to assess the purity of the fractions. (B) Scan of entire gels.
Figure S2. Evaluation of electrochemiluminescence-based (ECL) assay system. (A) The reactivity of antibodies against myelin and nonmyelin membranes was assessed using an electrochemiluminescence-based (ECL) assay system. Antibodies against myelin proteins (myelin basic protein, MBP; 2′,3′-cyclic nucleotide 3′-phosphodiesterase, CNP; myelin-oligodendroyte glycoprotein, MOG) revealed higher reactivity against myelin fraction as compared to the nonmyelin membrane. However, GFAP, an antibody against astrocytes, exhibited higher reactivity against nonmyelin membrane fraction as compared to myelin. The interassay variability ranged from 2% to 5% (SD/Mean × 100) based on 5 repeated measurements. (B) There is no correlation between ECL signal against myelin and IgG levels in the CSF from MS patients in the discovery cohort. (C) ECL-based liposome assay was validated using anti-GalC antibody as a positive control and anti-MOG antibody as a negative control. The interassay variability ranged from 2% to 4% (SD/Mean × 100) based on five repeated measurements.
Table S1. Summary of general characteristics of the studied subjects.
Table S2. Diagnoses of the patients with OND and OCB^+.
Table S3. Summary of CSF analysis.
Data S1. Supplementary methods.

