Journal of Neuroimmunology 336 (2019) 577021

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

The accuracy of flow cytometric cell-based assay to detect anti-myelin oligodendrocyte glycoprotein (MOG) antibodies determining the optimal method for positivity judgement

Kazuo Sugimoto[a,b], Masahiro Mori[a,*], Jia Liu[a,c], Satoru Tanaka[d], Kimihiko Kaneko[e,f], Satoru Oji[d], Toshiyuki Takahashi[e,g], Akiyuki Uzawa[a], Tomohiko Uchida[a], Hiroki Masuda[a], Ryohei Ohtani[a], Kyoichi Nomura[d], Takaki Hiwasa[h], Satoshi Kuwabara[a]

[a] Department of Neurology, Graduate School of Medicine, Chiba University, Chiba, Japan
[b] Department of Neurology, Dongzhimen Affiliated Hospital, Beijing University of Chinese Medicine, Beijing, China
[c] Department of Neurology, The Second Hospital of Hebei Medical University, Shijiazhuang, Hebei, China
[d] Department of Neurology, Saitama Medical Center, Saitama Medical University, Kawagoe, Japan
[e] Department of Neurology, Tohoku University Graduate School of Medicine, Sendai, Japan
[f] Department of Neurology, National Hospital Organization Miyagi National Hospital, Miyagi, Japan
[g] Department of Neurology, National Hospital Organization Yonezawa National Hospital, Yonezawa, Japan
[h] Department of Biochemistry and Genetics, Graduate School of Medicine, Chiba University, Chiba, Japan

ARTICLE INFO

Keywords:
Myelin oligodendrocyte glycoprotein
Neuromyelitis optica spectrum disorders
Multiple sclerosis
Flow cytometry
Aquaporin-4
Cell-based assay
Autoantibody
Anti-MOG antibody-associated disorders
Anti-AQP4 antibody-associated disorders

ABSTRACT

To illustrate the accuracy of the fluorescence-activated cell sorting cell-based assay (FACS-CBA) and to detect anti-myelin oligodendrocyte glycoprotein (MOG) antibodies and ascertain the optimal method for positivity judgement, referencing the findings of microscopic CBA. We tested serum anti-MOG antibodies in 57 patients with central nervous system inflammatory disorders (CIDs), 30 healthy controls (HCs), and 63 disease controls (DCs) by FACS-CBA. To assess the diagnostic performance of 2 positive judgement methods for FACS-CBA, we evaluated the ratio of positive cells (RPC) and median fluorescence intensity (MFI_{ratio}); samples from 57 CIDs and 3 antiaquaporin-4 antibody-positive patients whose anti-MOG antibody levels were relatively high but negative by FACS-CBA were tested by microscopic CBA. Blinded to the RPC and MFI_{ratio} results, we classified the acquired dot plot into 3 patterns—"upright," "broadband," and "oblique"—as pattern analysis. The sample with the highest RPC in CIDs was subjected to serial dilution analysis. Finally, we analyzed the clinical and laboratory data of anti-MOG antibody-positive patients in the acute phase. Referencing results by microscopic CBA and receiver-operating characteristic curve analysis, the area under the curve, sensitivity, specificity, and cutoff value were 0.952, 92%, 94%, and 1.52 for RPC and 0.931, 79%, 94%, and 6.39 for MFI_{ratio}, respectively, suggesting the optimality of RPC for positive judgement. Titers by microscopic CBA analysis significantly correlated with RPC ($P = .031$). In the validation study, the positive rate of RPC for anti-MOG antibodies was 42.1% in CIDs, but 0% in HCs and DCs (both $P < .001$). In the pattern analysis, all anti-MOG antibody-positive patients but none of the HCs and DCs exhibited the "oblique" pattern. Serial dilution curve analysis fit a quaternary polymodal. FACS-CBA using RPC analysis for anti-MOG antibodies displayed relatively higher specificity, sensitivity, and semiquantitative property, indicating it could become another acceptable test to detect anti-MOG antibodies.

1. Introduction

Neuromyelitis optica spectrum disorders (NMOSD) is an inflammatory disorder of the central nervous system (CNS), especially the optic nerve and spinal cord (Wingerchuk et al., 2007). While serum autoantibodies against aquaporin-4 (AQP4), which exists profusely on the foot process of astrocytes, are noted in 60%–80% of patients with NMOSD, those against myelin oligodendrocyte glycoprotein (MOG), which exists on the myelin sheath on the oligodendrocyte, are reported in approximately 10% of patients with NMOSD (Sato et al., 2014;

* Corresponding author at: Department of Neurology, Graduate School of Medicine, Chiba University, 1-8-1, Inohana, Chuo-ku, Chiba 260-8670, Japan.
E-mail address: morim@faculty.chiba-u.jp (M. Mori).

https://doi.org/10.1016/j.jneuroim.2019.577021
Received 22 April 2019; Received in revised form 21 July 2019; Accepted 13 August 2019

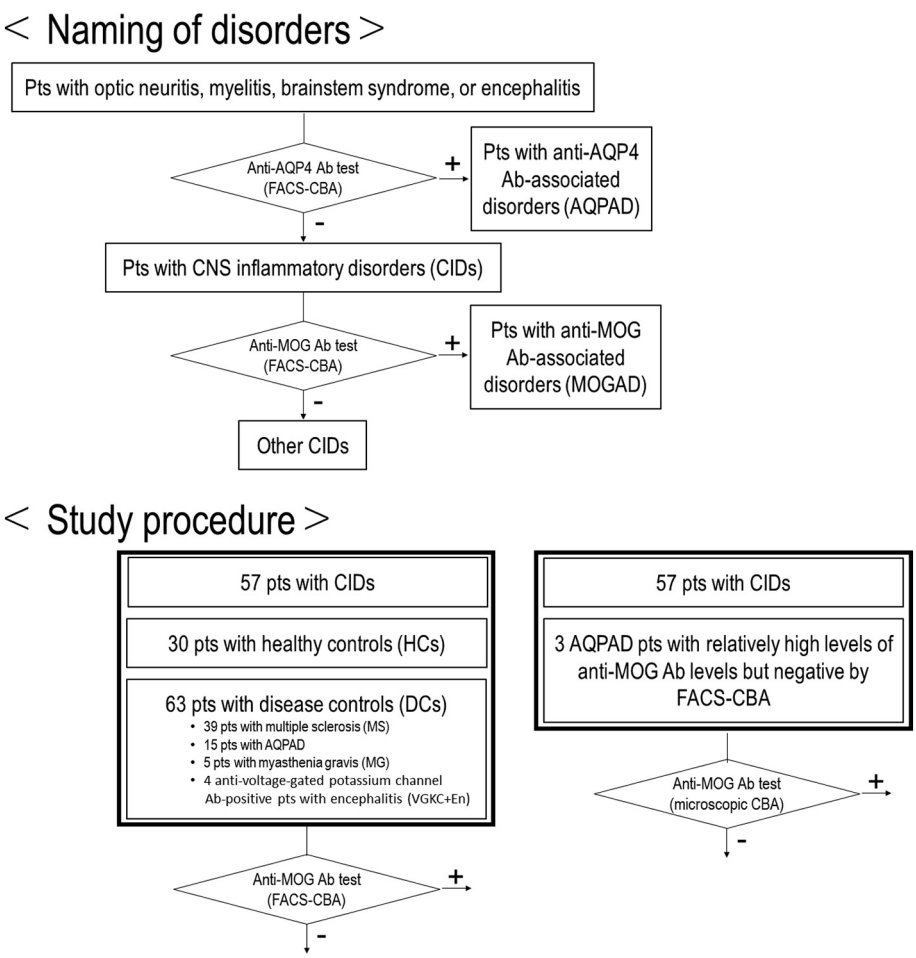


Fig. 1. Naming of disorders and study procedure.
AQP4, aquaporin-4; FACS-CBA, fluorescence-activated cell sorting cell-based assay; CNS, central nervous system; CIDs, central nervous system inflammatory disorders; MOG, myelin oligodendrocyte glycoprotein; AQPAD, anti-AQP4 antibody associated disorders; MOGAD, anti-MOG antibody-associated disorders; HCs, healthy controls; DCs, disease controls.

Hamid et al., 2017).

MOG is a membrane protein that is expressed only on the outermost layer of the myelin sheath and oligodendrocyte cytoplasmic membrane. Anti-MOG antibodies are found in not only NMOSD but also other CNS inflammatory disorders (CIDs), including optic neuritis, myelitis, brainstem syndrome, acute disseminated encephalopathy, cortical encephalitis, seizures and atypical multiple sclerosis (MS) cases (Ogawa et al., 2017; Spadaro et al., 2016). However, to date, the function of anti-MOG antibodies remains unclear. Moreover, whether an antibody is pathogenic or not remains debatable. Nevertheless, some studies have suggested that the antibody induces the complement-dependent cytotoxic effect by the antigen–antibody complex in the CNS (Peschl et al., 2017). Perhaps anti-MOG antibodies are more common in patients with binocular optic neuritis who lack anti-AQP4 antibodies (Sato et al., 2014; Hamid et al., 2017). Meanwhile, the existence of anti-AQP4 and anti-MOG double-positive patients also remains debatable, if they truly exist, probably as a technical artifact in most cases (Di Pauli et al., 2015). As mentioned earlier, the anti-MOG antibody-positive and anti-AQP4 antibody-positive groups exhibited similar but differentiated characteristics in clinical symptoms, radiological characteristics, and prognosis, necessitating the precise identification of these 2 antibodies.

At present, microscopic CBA and enzyme-linked immunosorbent assay (ELISA) are extensively applied high-specificity methods to identify autoantibodies in scientific and clinical antibody testing; microscopic CBA is considered to be more sensitive than ELISA (Apiwattanakul et al., 2012). The 2015 International Consensus Diagnostic Criteria for NMOSD strongly recommended microscopic CBA (Wingerchuk et al., 2015; Kim et al., 2017). Lately, flow cytometry (fluorescence-activated cell sorting [FACS]) live cell-based assay (CBA) technique, a quantitative detection technique based on the CBA technology, is increasingly applied in various autoantibodies identification, including anti-AQP4 antibody testing. In a study, FACS-CBA for anti-AQP4 antibodies detection in patients with NMOSD exhibited that its sensitivity and specificity corroborated those of microscopic CBA (Majed et al., 2016). Compared with ELISA technology, CBA, including FACS-CBA and microscopic CBA, is more suitable for detecting specific antibodies in autoimmune diseases (Westphal et al., 1994; Waters et al., 2016), as a posttranslational modification in CBA is similar with the one in humans. In FACS-CBA, regarding the methods of data analysis and positivity judgement, the median fluorescence intensity ratio (MFI_{ratio}) was conventionally used for positive or negative results judgement, and the ratio of positive cells (RPC) was primarily applied in the study of inflammatory factors, including surface antigens and intracellular cytokines. Notably, to date, an optimal method for data and results analysis for autoantibodies, including anti-MOG antibodies in autoimmune diseases in the CNS, remains debatable and unestablished, rendering our study even more urgent and necessary. Hence, this study aims to illustrate the accuracy of FACS-CBA to detect anti-MOG antibodies and ascertain the optimal method for positivity judgement, referencing the findings of microscopic CBA.

2. Materials and methods

2.1. Serum samples and naming of disorders

We collected serum samples from 57 consecutive outpatients and inpatients who had optic neuritis, myelitis, brainstem syndrome, or encephalitis, whose physician suspected anti-MOG antibodies and requested us to test anti-MOG antibodies and the written patients' consent was obtained at meantime. The study was approved by the Ethics Committee of Chiba University. Before anti-MOG antibody measurement, we measured anti-AQP4 antibodies using FACS-CBA as described later. If the result for anti-AQP4 antibodies was positive, the patients' disorder was named anti-AQP4 antibody-associated disorder (AQPAD); however, if it was negative, we named "chronic inflammatory disorders other than AQP4-IgG-positive NMOSD" as CIDs. Finally, we tested anti-MOG antibodies in serum samples from 57 patients with CIDs (acute phase: 32 samples; chronic phase: 25 samples), 30 healthy controls (HCs), and 63 disease controls (DCs), including 15 patients with AQPAD (comprising 14 NMOSD), 39 with MS, 5 with myasthenia gravis (MG), and 4 anti-voltage-gated potassium channel antibody-positive patients with encephalitis (VGKC + En) by FACS-CBA, as described later and the clinical information was blinded to the clinical information to one of the co-authors (KS; Fig. 1). In 57 patients with CIDs, 37 patients who had never been tested for anti-MOG antibodies were seen at Chiba University Hospital (Chiba, Japan) and 20 at Saitama Medical Center (Kawagoe, Japan) between 1996 and 2017. All DCs were examined at Chiba University Hospital from 2001 to 2017. Eleven of the 57 patients with CIDs and 10 (5 AQPAD and 5 MS) of the 63 DCs were treated with oral prednisolone treatment at serum sampling. "Attack" was defined as new, recurring, or worsening when neurological symptoms were persistent for at least 24 h, in the absence of fever or infection. "Acute phase" was defined as the period within 1 month of the attack, whereas "chronic phase" was defined as the period at least 1 month after the attack. All samples in the attack phase were obtained before the attack-related treatment. While all patients with NMOSD fulfilled the 2015 International Consensus Diagnostic Criteria (Wingerchuk et al., 2015), patients with MS fulfilled the 2017 McDonald's Diagnostic Criteria (Thompson et al., 2018). Of note, all samples were stored at −80 °C until assayed. If anti-MOG antibodies were judged to be positive, we named the patients' disorder as anti-MOG antibody-associated disorder (MOGAD).

2.2. Live CBAs using FACS

We transfected 1×10^6 human embryonic kidney (HEK293) cells using Neon (Thermo Fisher Scientific K.K., Waltham, MA, USA) with 5 μg of plasmid DNA (pIRES2-Aequorea coerulescens green fluorescent protein/human full-length *MOG* or *AQP4* isoform gene). Next, the cells were cultured for additional 48 h posttransfection and lifted by incubation with phosphate-buffered saline (PBS) containing 0.02% ethylenediamine tetraacetic acid (EDTA) and 0.1% trypsin for 5 min at room temperature. The subsequent steps were performed at 4 °C. We resuspended the cells in PBS containing 0.02% sodium azide (pH 7.2), 0.5% bovine serum albumin (BSA), and 2-mM EDTA; rotated for 10 min; diluted in PBS containing 2% BSA, 10% normal goat serum, 15-mM EDTA, and 0.05% sodium azide; and dispensed into a 1.5 ml EP tube. Subjects' sera were diluted in blocking buffer (PBS containing 2% BSA, 10% normal goat serum, 15-mM EDTA, and 0.05% sodium azide), and added to 1.5 ml EP tube in duplicated at a 1:5 final dilution. After incubation while shaking the plates (30 min, 300 rpm, 4 °C), we washed the cells 3 times with PBS. Anti-allophycocyanin (APC)-conjugated human-IgG1 antibody (Miltenyi Biotec) was added at 1:20 diluted in blocking buffer (PBS containing 2% BSA, 10% normal goat serum, 15-mM EDTA, and 0.05% sodium azide). Of note, the application of anti-human-IgG1 antibody was based on the study which reported FL-MOG and restricting to IgG1-Abs substantially improves specificity for non-MS demyelinating diseases (Waters et al., 2015). After shaking (30 min, 300 rpm, 4 °C), we washed the cells 3 times with PBS. Of note, all washed used centrifugation steps were conducted for 10 min at 3000 rpm in a conventional centrifuge at 4 °C. (RXII, Ltd., headquartered in Tokyo, Japan) Finally, we fixed all samples in 4% paraformaldehyde and analyzed by flow cytometer (BD FACS Canto II; BD Biosciences, San Jose, CA, USA) using FlowJo® software 10.4.1 (FlowJo LLC, Ashland, OR, USA). The gating strategy is shown in Supplemental Fig. Intact live HEK293 cells were gated to exclude cell aggregates based on forward scatter (FSC) and side scatter (SSC) plot (Supplemental Fig-A).

2.3. Two analysis methods: RPC and MFI_{ratio} analysis

We performed two analysis methods for positivity judgement after selecting live cells as follows: (a) to assess the number of positive cells in the transfected cell population (RPC method, Supplemental Fig-B); (b) the MFI_{ratio} analysis (Supplemental Fig-C). The RPC analysis involves evaluating the percentage of APC-conjugated IgG-binding cells in live green fluorescent protein $(GFP)^+$ cells. The cut-off value of APC fluorescence intensity for RPC analysis was determined as the mean plus 10 standard deviations (=1.53) of the values in 30 healthy controls. The MFI_{ratio} analysis involves evaluating the MFI_{ratio} of the APC channel in live GFP^+ cells. We set an acquisition gate for the GFP-MOG–positive population and measured the MFI_{ratio} of the APC fluorescence. Then, we gated two populations based on the GFP expression as follows: positive (high MOG expression) and negative (low/no MOG expression). The median APC fluorescence intensity for the GFP^+ population suggested the relative abundance of human IgG potentially bound to MOG surface epitopes; the MFIratio for the GFP^- population suggested nonspecifically bound IgG. Furthermore, we evaluated the IgG binding index as the ratio of the average MFIratio for duplicate aliquots of each cell population ($MFIratio\text{-}GFP^+$/$MFIratio\text{-}GFP^-$). FACS analysis using RPC and MFI_{ratio} methods were performed in parallel using the same thawed sample.

2.4. Microscopic CBA

After measuring anti-MOG antibodies by flow cytometry and performing the RPC and MFIratio analyzes, one of the authors (TT), who was blinded to the FACS-CBA results, measured anti-MOG antibodies using microscopic CBA, as described previously (Sato et al., 2014), for samples from 57 patients with CIDs and 3 with AQPAD with relatively high RPC and/or MFI_{ratio} levels of anti-MOG antibodies but negative by FACS-CBA. The serum was screened at a 1:128 dilution comparing the staining with vector- and MOG-transfected cells; if positive, retested at 1:128 dilution titrated further in 2-fold dilution steps by one of the authors (KK), who was also blinded to the FACS-CBA results (Sato et al., 2014). Of note, the results of the microscopic CBA were blinded to the FACS-CBA tester (KS; Fig. 1).

2.5. Receiver-operating characteristic curve analysis to determine the optimality of RPC and MFI_{ratio} analyzes

We used the judgement results of seropositivity for anti-MOG antibodies by microscopic CBA as a reference standard to determine the optimality of the RPC and MFIratio analyzes. In addition, we conducted the receiver-operating characteristic (ROC) curve analysis using data of samples from 57 patients with CIDs and 3 anti-AQP4 antibody-positive patients described earlier. Furthermore, the optimal cutoff point was decided automatically to obtain the highest sum of sensitivity and specificity.

2.6. Validation of the sensitivity and specificity of the RPC analysis method for anti-MOG antibodies using sera from CIDs, HCs, and DCs

We compared the results by the RPC and MFIratio analysis methods for anti-MOG antibodies using sera from 57 CIDs, 30 HCs, and 63 DCs, as in the "Serum samples and naming of disorders" section, to validate the efficacy of the RPC analysis method for anti-MOG antibodies using larger samples, including HCs and DCs. Finally, the cutoff was evaluated by the mean plus 10 standard deviation.

2.7. Pattern analysis of cell imaging after smoothing

Smoothing is an extensively used tool in the analysis of flow cytometry data, as it makes discerning a trend easier. After selecting transfected GFP^+ cells (Supplemental Fig-D), we performed smoothing (Supplemental Fig-F) to dot plot cells (Supplemental Fig-E), and one of the authors (MM), who was blinded to the RPC and MFIratio results, classified the cell imaging into the following 3 patterns: "upright," "broadband," and "oblique." The "broadband" and "upright" patterns are similar, but we defined the pattern as "broadband" when the highest value of the APC fluorescence intensity after smoothing was $> 2 \times 10^2$.

2.8. Serial dilution curve analysis

We serially diluted the sample that exhibited the highest RPC in CIDs. If the original concentration of anti-MOG antibodies was A, dilution samples were prepared from 1/5A to 1/3125A. Then, anti-MOG antibodies were measured in the diluted samples using FACS-CBA and analyzed by the RPC method as described earlier.

2.9. Clinical characteristics of patients with MOGAD in the acute phase judged by RPC analysis method

In this study, we elucidated the clinical characteristics of patients with MOGAD in the acute phase judged by RPC analysis, including age, female ratio, the proportion of patients having optic neuritis, simultaneous bilateral optic neuritis, myelitis, long-cord lesion > 3 vertebral segments, brainstem syndrome with intractable hiccup, and encephalitis diagnosed with acute disseminated encephalomyelitis, compared with AQPAD patients.

2.10. Statistical analysis

Statistical analyzes conducted to evaluate the sensitivity and specificity of each method of data analysis were showed as: Sensitivity = True positive / (True positive + False negative); specificity = True negative / (False positive + True negative). We used the Mann–Whitney *U* test to compare differences between two independent groups and the Pearson or Spearman correlation analysis for correlation analysis. Values of $P < .05$ were considered to indicated statistical significance. All data sets were analyzed using SPSS 21.0 Software.

3. Results

3.1. Demographic data of CIDs, HCs, and DCs

Table 1 summarizes the demographic data of CIDs, HCs, and DCs. The age at serum sampling and female ratio exhibited no intergroup difference among CIDs, HCs, and DCs. In CIDs, HCs, and DCs, the median (interquartile range [IQR]) age was 42.0 (31.0–53.0), 35.0 (26.5–38.0), and 43.0 (35.5–60.5) years, and the female ratio was 59.6%, 56.7%, and 69.8%, respectively. In CIDs, 30 (52.6%), 16 (28.1%), 7 (12.3%), 2 (3.5%), and 2 (3.5%) patients had a lesion in the optic nerve, spinal cord, cerebrum, brainstem, and no record in clinical data respectively, at the disease onset. During their disease courses, 30 (52.6%) patients had optic neuritis, of whom 9 (15.8%) had simultaneous bilateral optic neuritis, 16 (28.1%) had myelitis, and 9 (15.8%) had long-cord lesion > 3 vertebral segments. Furthermore, 6 patients with CIDs (10.5%) were diagnosed with acute disseminated encephalomyelitis.

Table 1
Demographic data of CIDs, HCs, and DCs.

	CIDs	HCs	DCs
	n = 57	*n* = 30	*n* = 63
Female, *n* (%)	34 (59.6)	17 (56.7)	44 (69.8)
Age, years[a]	42.0 (22.0)	35.0 (11.5)	43.0 (25.0)
Lesion of disease onset			
Optic nerve, *n* (%)	30 (52.6)		
Bilateral optic neuritis, *n* (%)	9 (15.8)		
Spinal cord, *n* (%)	16 (28.1)		
> 3 vertebral segments, *n* (%)	9 (15.8)		
Cerebrum, *n* (%)	7 (12.3)		
Brainstem, *n* (%)	2 (3.5)		
No record in clinical data, *n* (%)	2 (3.5)		

CIDs, central nervous system inflammatory disorders; HCs, healthy controls; DCs, disease controls.
[a] Data are presented as medians (interquartile range).

3.2. ROC curve analysis to ascertain the optimality of RPC and MFI_{ratio} analyzes

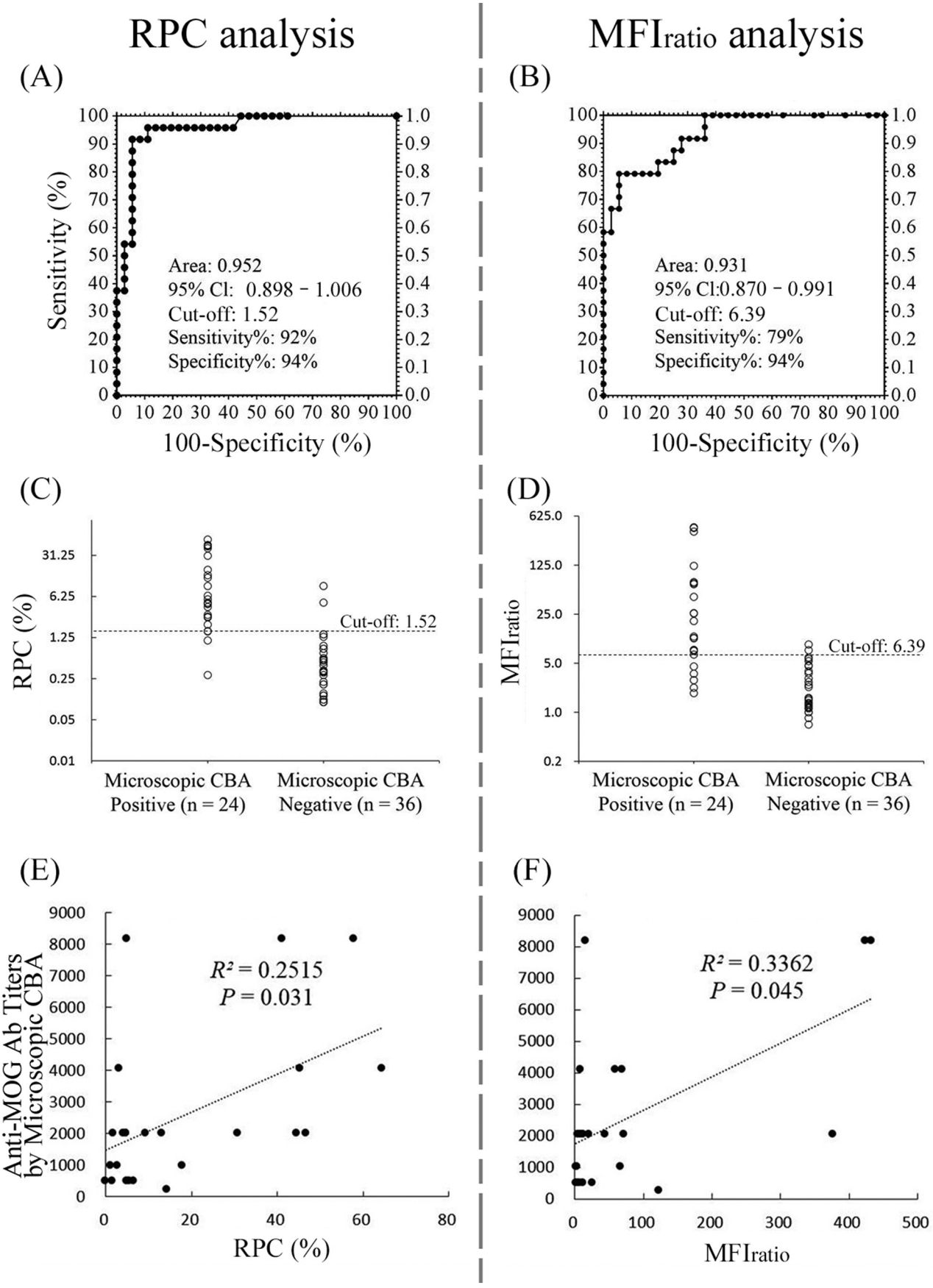
The ROC for the RPC and MFIratio analyzes revealed that the areas under the curves were 0.952 and 0.931, respectively (Fig. 2A and B). In addition, among 60 serum samples obtained from 57 patients with CIDs and 3 anti-AQP4 antibody-positive patients with relatively high RPC and/or MFI_{ratio} levels of anti-MOG antibodies but negative by FACS-CBA, RPC and MFIratio analyzes exhibited a sensitivity of 92% and 79%, respectively. Both assays achieved a superior specificity of 94% with a cutoff value of 1.52 for the RPC analysis and of 6.39 for the MFIratio analysis (Fig. 2C and D).

3.3. Correlation of anti-MOG antibody titers between microscopic CBA and FACS-CBA with RPC and MFI_{ratio} analyzes

Fig. 3 (bottom) reveals the correlation of anti-MOG antibody titers between microscopic CBA and FACS-CBA with RPC (Fig. 2E) and MFIratio (Fig. 2F) analyzes in 24 anti-MOG antibody-positive patients (acute phase, 19 patients; chronic phase, 5 patients). The titers by the microscopic CBA analysis moderately correlated with FACS-CBA with RPC ($P = .031$) and with MFIratio ($P = .045$) analyzes. Furthermore, the correlation with RPC was stronger than that with MFIratio.

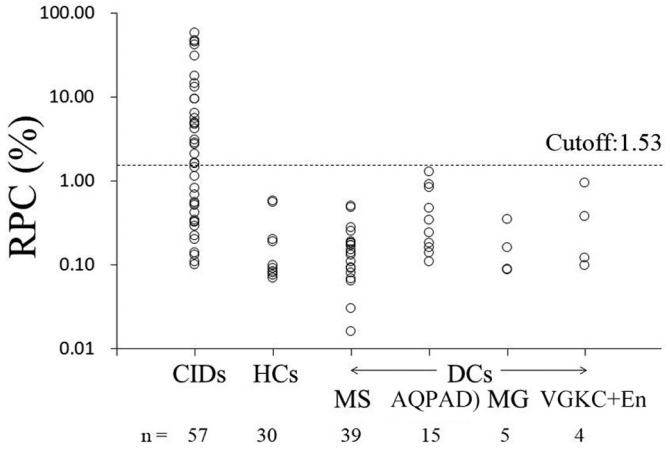
3.4. Validation of the sensitivity and specificity of RPC analysis method for anti-MOG antibodies using sera from CIDs, HCs, and DCs

In this study, we measured anti-MOG antibodies using sera from 57 CIDs, 30 HCs, and 63 DCs to validate the sensitivity and specificity of RPC analysis method (Fig. 3). Of 57 patients with CIDs, 24 (42.1%) were positive for anti-MOG antibodies after the RPC analysis; however, all 30 HCs and 63 DCs were negative for anti-MOG antibodies. The positive rate was significantly higher in CIDs than in HCs and DCs (both $P < .001$). In CIDs, HCs, and DCs, the median (IQR) RPC was 0.56 (0.00–4.99), 0 (0.00–0.08), and 0.09 (0.00–0.18), respectively. The RPC was significantly higher in CIDs than in HCs and DCs (both $P < .001$). Furthermore, the cutoff value of 1.52 evaluated by the ROC analysis was about the mean plus 10 standard deviations obtained from 30 HCs.



(caption on next page)

Fig. 2. Comparisons between two analysis methods of fluorescence-activated cell sorting cell-based assay (FACS-CBA) assay.
Referencing the results of microscopic CBA, we compared the diagnostic performance of the ratio of positive cells (RPC; A, C, and E) and median fluorescence intensity ratio (MFIratio; B, D, and F). A and B, Results of the receiver-operating characteristic (ROC) curve analysis. C and D, anti-MOG antibody levels in microscopic CBA-positive and -negative central nervous system inflammatory disorders (CIDs) using the RPC (C) and MFIratio (D), respectively. E and F, Correlations of titers using microscopic CBA with RPC and MFIratio.



		Microscopic CBA	
		Positive	Negative
FACS-CBA RPC	Positive	22	2
	Negative	2	34
Sensitivity(%)		92%	
Specificity(%)		94%	

Fig. 3. Anti-myelin oligodendrocyte glycoprotein (MOG) antibody levels measured by the ratio of positive cells (RPC) method among groups.
Top, comparison of RPC among central nervous system inflammatory disorders (CIDs), healthy controls (HCs), and disease controls (DCs), including multiple sclerosis (MS), anti-AQP4 antibody associated disorders (AQPAD), myasthenia gravis (MG), and anti-voltage-gated potassium channel antibody-positive encephalitis (VGKC + En). Bottom, correlation between the RPC results by FACS-CBA and microscopic CBA, and the sensitivity and specificity of RPC by FACS-CBA when the results of microscopic CBA are true values.

3.5. Pattern analysis of cell imaging after smoothing

Fig. 4 shows the representative results of the pattern analysis. All our FACS results in the validation study described earlier were also classified into three patterns. All patients with MOGAD but none of the HCs and DCs exhibited the “oblique” pattern. All HCs, MS, and MG patients in DCs exhibited the “upright” pattern. In addition, three of 15 AQPAD and 1 of 4 VGKC + En exhibited the “broadband” pattern and other AQPAD or VGKC + En patients exhibited the “upright pattern.”

3.6. Serial dilution curve analysis

Fig. 5 presents the results of the serial dilution curve for anti-MOG antibodies, which approximates the quadratic equation as follows: $y = 3E\text{–}08 \times 4 \quad - \quad 9E\text{–}07 \times 3 \quad - \quad 4E\text{–}06 \times 2 + 0.0004 \times + 5E\text{–}05$ ($R^2 = 1$), implying that we can semi-quantify the volume of anti-MOG antibodies within a certain range by solving the quadratic equation.

3.7. Clinical characteristics of patients with MOGAD in the acute phase judged by the RPC analysis

Table 2 presents the clinical data of 19 patients with MOGAD and 12 patients with AQPAD in the acute phase. The seropositivity was ascertained by RPC analysis. Among the patients with MOGAD, the female ratio was 47.4%, and the median (IQR) age was 38.1 (42.3) years, which was lower than that of patients with AQPAD (median, 61.8; IQR, 31.9). The median (IQR) Expanded Disability Status Scale (EDSS) score was 2.0 (1.8), which was significantly lower than that of patients with AQPAD (median, 5.0; IQR, 4.1). The lesion site accountable for the acute attack was MOGAD in patients who had a history of optic neuritis without symptoms, and signs suggesting other lesion sites were the brain and spinal cord, which were significantly more frequent in patients with MOGAD (73.7%) than in those with AQPAD (25.0%). Other parameters, including the disease duration, attack number, cells, and proteins in the cerebrospinal fluid (CSF) exhibited no difference between the two groups. The RPC for anti-MOG antibodies positively correlated with the CSF-protein levels ($P = .017$; Fig. 6). Other analyzed parameters, including age, disease duration, EDSS, attack number, and CSF cells, exhibited no correlation with the RPC.

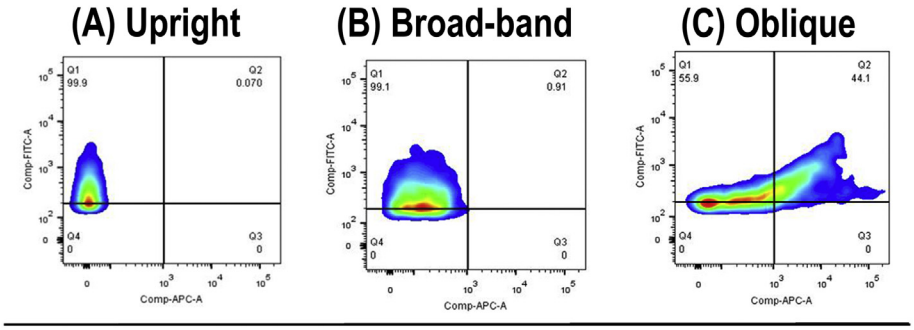
3.8. Four cases with inconsistent results between FACS-CBA and microscopic CBA

Fig. 7 shows the four cases with inconsistent results between FACS-CBA and microscopic CBA. Case 1 and 2 were positive for MOG-IgG by FACS-CBA but negative by microscopic CBA. Case 3 and 4 were negative for MOG-IgG by FACS-CBA but positive by microscopic CBA. All the four cases were not so different from previously reported clinical features of MOG-IgG-positive CIDs; absence of female predominance, frequency of optic neuritis and/or myelitis, and not so high disability.

4. Discussion

First, this study established the anti-MOG antibody assay using FACS-CBA. The two primary methods to judge antibody positivity using FACS are RPC and MFIratio. Using the ROC curve, our data revealed that the RPC method for the anti-MOG antibody test is better than the MFIratio method. The sensitivity was similar between the RPC and MFIratio analysis methods, but the specificity was higher in the RPC method.

In this study, the anti-MOG antibodies results obtained by FACS-CBA corroborated those obtained by microscopic CBA, and the titers of anti-MOG antibodies by microscopic CBA positively correlated with the RPC by FACS-CBA. However, some differences were noted between the results of FACS-CBA and microscopic CBA. In 57 CIDs tested by microscopic and FACS-CBA assays, 2 patients were positive by FACS-CBA assay but negative by microscopic CBA, and other 2 patients were positive by microscopic CBA assay but negative by FACS-CBA. Whether FACS-CBA or microscopic CBA is a better method to judge autoantibodies remains debatable (Fig. 7). Regarding the anti-AQP4 antibody test, CBA is usually considered superior to ELISA. However, the results of microscopic CBA are interpreted by visually observing the immunofluorescence signals with a microscope, which could be highly observer-dependent and lack objective quantification of the results (Yang et al., 2016). Regarding anti-MOG antibodies, which of FACS-CBA or microscopic CBA is superior remains unclear. These assays may be able to make up for each other's weak points.



		(A) Upright	(B) Broad-band	(C) Oblique
HC (n=30)				
CIDs (n=57)	MOGAD (n=24)	0	0	24
	Others (n=33)	33	0	0
DCs (n=63)	AQPAD (n=15)	12	3	0
	MS (n=39)	39	0	0
	MG (n=5)	5	0	0
	VGKC+En (n=4)	3	1	0

Fig. 4. Pattern analysis of myelin oligodendrocyte glycoprotein (MOG)-expressing cells stained with patients' sera after the smoothing process by flow cytometry.
The pattern of the dot plot of MOG-expressing cells stained with patients' sera after the smoothing process was classified into the following three groups: (A) upright, (B) broadband, and (C) oblique. The table in the figure presents the correlation between the clinical phenotype (healthy controls [HCs]), central nervous system inflammatory disorders (CIDs), including anti-MOG antibody-associated disorders (MOGAD), anti-AQP4 antibody-associated disorders (AQPAD), and others and the 3 patterns.

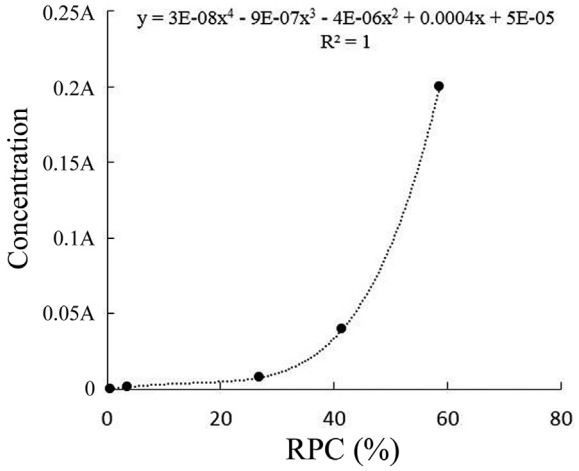


Fig. 5. Serial dilution curve analysis.
We serially diluted samples that exhibited the highest ratio of positive cells (RPC) in central nervous system inflammatory disorders (CIDs). If the original concentration of anti-MOG antibodies was A, the dilution samples were made from 1/5A to 1/3125A. Then, anti-MOG antibodies were measured in the diluted samples using flow cytometric cell-based assay (FACS-CBA) and analyzed by the RPC method as described earlier. The serial dilution curve approximates the quadratic equation.

In this study, the pattern analysis of the dot plot could facilitate judging the positivity of anti-MOG antibodies. Perhaps the "oblique" pattern implies positivity because it suggests the existence of double-positive cells with GFP and the anti-MOG antibody binding to MOG. In addition, 2 patients were positive by the FACS-CBA assay but negative by microscopic CBA, and other 2 patients were positive by microscopic CBA assay but negative by FACS-CBA in 57 patients with CIDs; the former 2 patients who were single-positive for microscopic CBA exhibited the "upright" pattern, and the latter 2 exhibited the "oblique" pattern. Although this might suggest the superiority of FACS-CBA in this assay, further well-organized, extensive studies using large sample sizes are warranted to ascertain which assay is better in both sensitivity and specificity. Moreover, the serial dilution curve analysis in this study suggested that we could semi-quantify the volume of anti-MOG antibodies using the fourth approximate expression within a certain range.

Table 2
Clinical characteristics of patients with MOGAD and AQPAD in the acute phase.

	MOGAD	AQPAD	*P*
	n = 19	*n* = 12	
Female, *n* (%)	9.0 (47.4)	9.0 (75.0)	N.S.
Age, years[a]	38.1 (42.3)	61.8 (31.9)	0.049
Disease duration, month[a]	0.7 (18.3)	2.4 (45.1)	N.S.
EDSS[a]	2.0 (1.8)	5.0 (4.1)	0.037
Attack number[a]	0.0 (1.0)	0.5 (1.3)	N.S.
CSF cells[a]	9.0 (40.0)	15.0 (22.0)	N.S.
CSF proteins[a]	44.0 (33.0)	61.0 (40.5)	N.S.
History of clinical symptoms			
Optic neuritis only, *n* (%)	14 (73.7)	3 (25.0)	0.012
Other, *n* (%)	5 (26.3)	9 (75.0)	

AQPAD, anti-AQP4 antibody-associated disorders; CSF, cerebrospinal fluid; EDSS, Expanded Disability Status Scale; MOGAD, anti-MOG antibody-associated disorders; N.S., not significant.
[a] Data are presented as medians (interquartile range).

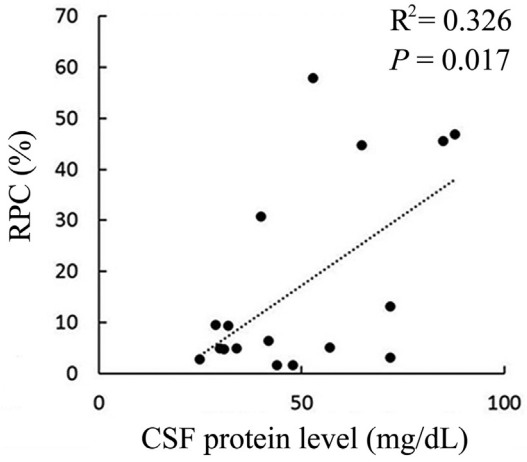


Fig. 6. Correlations between the ratio of positive cells (RPC) for anti-myelin oligodendrocyte glycoprotein (MOG) antibody levels and protein levels of the cerebrospinal fluid (CSF) in anti- MOG antibody-associated disorders (MOGAD).

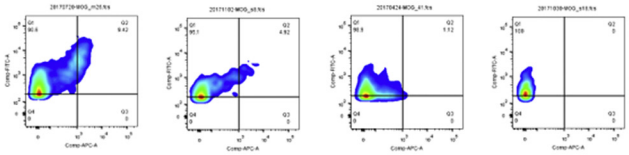
Case No.	(1)	(2)	(3)	(4)
MOG-IgG by FACS-CBA (RPC, %)	positive (9.42)	positive (4.92)	negative (1.12)	negative (0.00)
MOG-IgG by Microscopic CBA (Dilution)	negative	negative	positive (1024)	positive (512)
Pattern analysis of cell imaging after smoothing (FACS-CBA)				
Phase at serum sampling	attack phase	attack phase	attack phase	remission phase
Sex	F	M	F	M
Age	69	15	46	37
Disease duration (month)	0.5	0	0	216
EDSS	0.0	3.0	1.0	0.0
Total attack number	1	1	1	4
Location of lesions at the 1st attack	optic neuritis	myelitis	optic neuritis	optic neuritis
Locations of lesions during total disease course	optic neuritis	myelitis	optic neuritis	optic neuritis
Treatment at disease sampling	None	None	None	None
CSF IL-6 (pg/mL)	N.A.	N.A.	3.7	N.A.

Fig. 7. Four cases with inconsistent results between FACS-CBA and microscopic CBA.
Case 1 and 2 were positive for MOG-IgG by FACS-CBA but negative by microscopic CBA. Case 3 and 4 were negative for MOG-IgG by FACS-CBA but positive by microscopic CBA.
CBA, cell-based assay; CSF, cerebrospinal fluid; EDSS, Expanded Disability Status Scale; FACS, flow cytometry; MOG, myelin oligodendrocyte glycoprotein; MOG-IgG, anti-MOG antibody; N.A., not available; RPC, ratio of positive cells.

Previously, limiting the dilution method has been extensively used to compare volumes of autoantibodies (Herman et al., 2008); however, the method does not facilitate the comparison of volumes of autoantibodies from different patients. Of note, our semi-quantification method could resolve the problem and detect some minute changes, which may enable us to treat MOGAD patients in a more delicate manner according to the titers of MOG-IgG.

To maintain the high specificity of FACS-CBA, in our opinion, setting the cutoff to judge the “broadband” pattern as negative is necessary; this pattern could be attributed to weak and nonspecific binding of patients' immunoglobulins to MOG. In addition, this pattern was found in some sera from other neuroinflammatory disorders, including AQPAD and VGKC+En, with autoantibodies against some CNS components, but not in the sera from MG patients with autoantibodies against muscle neuromuscular junction. Although further investigation is warranted, there could be a cross-reaction of autoantibodies with some CNS components and MOG, or those sera might be double-positive for antibodies against some CNS components and MOG.

Regarding the clinical characteristics of patients with MOGAD, compared with those of acute AQPAD patients, acute MOGAD patients in this study exhibited markedly lower EDSS scores, which corroborated a previous study that considered that patients with MOGAD tended to exhibit a milder clinical phenotype than patients with AQPAD and were prone to recovery (Yan et al., 2016). However, no marked correlation exists between MOG-IgG and EDSS in the acute phase, which might be due to inadequate data. In addition, we observed a positive correlation between MOG-IgG and the quantity of the cerebrospinal fluid protein in this study, suggesting that MOG-IgG correlated with infection and higher levels of inflammatory responses in the CNS.

This study has some limitations. First, the sample size is not sufficiently large. However, as MOGAD is considered a rare disorder, collecting a large number of samples in one institution is challenging. In this study, we compared the optimal of RPC and MFIratio analysis methods. However, MFI includes not only the MFIratio method but also others, including the mean fluorescence intensity. Thus, the best method in the analysis using FACS-CBA remains debatable. The serial dilution curve fits a quaternary polymodal. In ELISA, the 4-parameter logistic regression is extensively used; however, a study reported that a polynomial would be more appropriate in some cases (Yan et al., 2016). Hence, further studies are warranted to elucidate the cause of the good fit by the quaternary polymodal and how models fit best to the dilution curve.

In conclusion, this study establishes FACS-CBA to test and semi-quantify anti-MOG antibodies. We believe this technique could be used accurately and reliably to measure anti- MOG antibodies and be potentially applicable to test other autoantibodies in the serum, which could provide a basis for the precise diagnosis and guide targeted and more effective treatment for autoimmune diseases.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jneuroim.2019.577021.

Acknowledgments

We wish to thank Mr. Yui Hiwasa for his technical assistance.

Funding

This research did not receive any specific grant from funding agencies.

References

Apiwattanakul, M., Asawavichienjinda, T., Pulkes, T., Tantirittisak, T., Hemachudha, T., Horta, E.S., Jenkins, S.M., Pittock, S.J., 2012. Diagnostic utility of NMO/AQP4-IgG in evaluating CNS inflammatory disease in Thai patients. J. Neurol. Sci. 320, 118–120.

Di Pauli, F., Hoftberger, R., Reindl, M., Beer, R., Rhomberg, P., Schanda, K., Sato, D., Fujihara, K., Lassmann, H., Schmutzhard, E., Berger, T., 2015. Fulminant demyelinating encephalomyelitis: insights from antibody studies and neuropathology. Neurol Neuroimmunol. Neuroinflamm. 2, e175.

Hamid, S., Whittam, D., Mutch, K., Linaker, S., Solomon, T., Das, K., Bhojak, M., Jacob, A., 2017. What proportion of AQP4-IgG-negative NMO spectrum disorder patients are MOG-IgG positive? A cross sectional study of 132 patients. J. Neurol. 264, 2088–2094.

Herman, R.A., Scherer, P.N., Shan, G., 2008. Evaluation of logistic and polynomial models for fitting sandwich-ELISA calibration curves. J. Immunol. Methods 339, 245–258.

Kim, S.M., Kim, S.J., Lee, H.J., Kuroda, H., Palace, J., Fujihara, K., 2017. Differential diagnosis of neuromyelitis optica spectrum disorders. Ther. Adv. Neurol. Disord. 10, 265–289.

Majed, M., Fryer, J.P., McKeon, A., Lennon, V.A., Pittock, S.J., 2016. Clinical utility of testing AQP4-IgG in CSF: guidance for physicians. Neurol Neuroimmunol. Neuroinflamm. 3, e231.

Ogawa, R., Nakashima, I., Takahashi, T., Kaneko, K., Akaishi, T., Takai, Y., Sato, D.K., Nishiyama, S., Misu, T., Kuroda, H., Aoki, M., Fujihara, K., 2017. MOG antibody-positive, benign, unilateral, cerebral cortical encephalitis with epilepsy. Neurol Neuroimmunol. Neuroinflamm. 4, e322.

Peschl, P., Schanda, K., Zeka, B., Given, K., Bohm, D., Ruprecht, K., Saiz, A., Lutterotti, A., Rostasy, K., Hoftberger, R., Berger, T., Macklin, W., Lassmann, H., Bradl, M., Bennett, J.L., Reindl, M., 2017. Human antibodies against the myelin oligodendrocyte glycoprotein can cause complement-dependent demyelination. J. Neuroinflammation 14, 208.

Sato, D.K., Callegaro, D., Lana-Peixoto, M.A., Waters, P.J., de Haidar, J.F., Takahashi, T., Nakashima, I., Apostolos-Pereira, S.L., Talim, N., Simm, R.F., Lino, A.M., Misu, T., Leite, M.I., Aoki, M., Fujihara, K., 2014. Distinction between MOG antibody-positive and AQP4 antibody-positive NMO spectrum disorders. Neurology 82, 474–481.

Spadaro, M., Gerdes, L.A., Krumbholz, M., Ertl-Wagner, B., Thaler, F.S., Schuh, E., Metz, I., Blaschek, A., Dick, A., Bruck, W., Hohlfeld, R., Meinl, E., Kumpfel, T., 2016. Autoantibodies to MOG in a distinct subgroup of adult multiple sclerosis. Neurol Neuroimmunol. Neuroinflamm. 3, e257.

Thompson, A.J., Banwell, B.L., Barkhof, F., Carroll, W.M., Coetzee, T., Comi, G., Correale, J., Fazekas, F., Filippi, M., Freedman, M.S., Fujihara, K., Galetta, S.L., Hartung, H.P., Kappos, L., Lublin, F.D., Marrie, R.A., Miller, A.E., Miller, D.H., Montalban, X., Mowry, E.M., Sorensen, P.S., Tintore, M., Traboulsee, A.L., Trojano, M., Uitdehaag, B., Vukusic, S., Waubant, E., Weinshenker, B.G., Reingold, S.C., Cohen, J.A., 2018. Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria. Lancet Neurol. 17, 162–173.

Waters, P., Woodhall, M., O'Connor, K.C., Reindl, M., Lang, B., Sato, D.K., Jurynczyk, M., Tackley, G., Rocha, J., Takahashi, T., Misu, T., Nakashima, I., Palace, J., Fujihara, K., Leite, M.I., Vincent, A., 2015. MOG cell-based assay detects non-MS patients with inflammatory neurologic disease. Neurol Neuroimmunol. Neuroinflamm. 2, e89.

Waters, P., Reindl, M., Saiz, A., Schanda, K., Tuller, F., Kral, V., Nytrova, P., Sobek, O., Nielsen, H.H., Barington, T., Lillevang, S.T., Illes, Z., Rentzsch, K., Berthele, A., Berki, T., Granieri, L., Bertolotto, A., Giometto, B., Zuliani, L., Hamann, D., van Pelt, E.D., Hintzen, R., Hoftberger, R., Costa, C., Comabella, M., Montalban, X., Tintore, M., Siva, A., Altintas, A., Deniz, G., Woodhall, M., Palace, J., Paul, F., Hartung, H.P., Aktas, O., Jarius, S., Wildemann, B., Vedeler, C., Ruiz, A., Leite, M.I., Trillenberg, P., Probst, M., Saschenbrecker, S., Vincent, A., Marignier, R., 2016. Multicentre comparison of a diagnostic assay: aquaporin-4 antibodies in neuromyelitis optica. J. Neurol. Neurosurg. Psychiatry 87, 1005–1015.

Westphal, J.R., Boerbooms, A.M., Schalwijk, C.J., Kwast, H., De Weijert, M., Jacobs, C., Vierwinden, G., Ruiter, D.J., Van de Putte, L.B., De Waal, R.M., 1994. Anti-endothelial cell antibodies in sera of patients with autoimmune diseases: comparison between ELISA and FACS analysis. Clin. Exp. Immunol. 96, 444–449.

Wingerchuk, D.M., Lennon, V.A., Lucchinetti, C.F., Pittock, S.J., Weinshenker, B.G., 2007. The spectrum of neuromyelitis optica. Lancet Neurol. 6, 805–815.

Wingerchuk, D.M., Banwell, B., Bennett, J.L., Cabre, P., Carroll, W., Chitnis, T., de Seze, J., Fujihara, K., Greenberg, B., Jacob, A., Jarius, S., Lana-Peixoto, M., Levy, M., Simon, J.H., Tenembaum, S., Traboulsee, A.L., Waters, P., Wellik, K.E., Weinshenker, B.G., 2015. International consensus diagnostic criteria for neuromyelitis optica spectrum disorders. Neurology 85, 177–189.

Yan, Y., Li, Y., Fu, Y., Yang, L., Su, L., Shi, K., Li, M., Liu, Q., Borazanci, A., Liu, Y., He, Y., Bennett, J.L., Vollmer, T.L., Shi, F.D., 2016. Autoantibody to MOG suggests two distinct clinical subtypes of NMOSD. Sci. China Life Sci. 59, 1270–1281.

Yang, J., Kim, S.M., Kim, Y.J., Cheon, S.Y., Kim, B., Jung, K.C., Park, K.S., 2016. Accuracy of the fluorescence-activated cell sorting assay for the aquaporin-4 antibody (AQP4-Ab): comparison with the commercial AQP4-Ab assay kit. PLoS One 11, e162900.

Kazuo Sugimoto a PhD student in Department of Neurology, Graduate School of Medicine, Chiba University. He specializes neuroimmunology.

Masahiro Mori is an associate professor of Department of Neurology, Graduate School of Medicine, Chiba University. His research is centred on neuroiimunology including multiple sclerosis, and neuromyelitis optica spectrum disorder.

Jia Liu is a PhD student in Department of Neurology, Graduate School of Medicine, Chiba University. She specializes neuroimmunology.

Satoru Tanaka is an assistant professor of Department of Neurology, Saitama Medical Center, Saitama Medical University.

Kimihiko Kaneko is a neurologist and researcher of Department of Neurology, Tohoku University Graduate School of Medicine.

Satoru Oji is a lecturer of Department of Neurology, Saitama Medical Center, Saitama Medical University.

Toshiyuki Takahashi is a neurologist and researcher of Department of Neurology, Tohoku University Graduate School of Medicine.

Akiyuki Uzawa is an assistant professor of Department of Neurology, Graduate School of Medicine, Chiba University. His is research is centred on autoimmune diseases of the nervous system and myasthenia gravis.

Tomohiko Uchida is a neurologist in Department of Neurology, Graduate School of Medicine, Chiba University.

Hiroki Masuda is an assistant professor of Department of Neurology, Graduate School of Medicine, Chiba University.

Ryohei Ohtani is a neurologist in Department of Neurology, Graduate School of Medicine, Chiba University.

Kyoichi Nomura is a professor of Department of Neurology, Saitama Medical Center, Saitama Medical University.

Takaki Hiwasa is an associate professor of Department of Biochemistry and Genetics, Graduate School of Medicine, Chiba University.

Satoshi Kuwabara is a professor of Department of Neurology, Graduate School of Medicine, Chiba University.