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Quantitative assays for anti-aquaporin-4 antibody with subclass analysis in neuromyelitis optica

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Abstract

Background: To clarify the clinical relevance of anti-aquaporin-4 (anti-AQP4) antibody titers and immunoglobulin (IgG) subclass.

Methods: Using a bridging enzyme-linked immunosorbent assay (ELISA), a flow cytometric assay (FCMA) and an immunofluorescence assay (IFA) for anti-AQP4 antibodies, sera from 142 patients with multiple sclerosis (MS) as defined by the McDonald criteria (2005), 29 with neuromyelitis optica (NMO) who fulfilled the 1999 criteria, 19 with recurrent and/or longitudinally extensive myelitis (RM/LM), 86 with other non-inflammatory neurological diseases (OND) and 28 healthy controls (HC) were studied.

Results: Anti-AQP4 antibody positivity rates by IFA, FCMA, and ELISA were 41.4%, 51.7% and 48.3%, respectively, in NMO (1999) patients, and 0% in the OND and HC groups. Twenty-six MS patients (18.3%) were positive for the antibody; 17 met the 2006 NMO criteria, including positivity for anti-AQP4 antibody, and five had longitudinally extensive myelitis (LM). Among the cases with anti-AQP4 antibody detected by FCMA, IgG1, 2, 3, and 4 anti-AQP4 antibodies were found in 97.8%, 37.0%, 6.5% and 6.5% respectively. There was no association of either antibody positivity or level of anti-AQP4 antibody IgG subclasses with clinical parameters after adjustment of p values for multiple comparisons.

Conclusions: FCMA and bridging ELISA are useful for detecting and quantifying anti-AQP4 antibodies.

Keywords

Aquaporin-4, neuromyelitis optica, multiple sclerosis, antibody, IgG, flow cytometry, ELISA

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Introduction

Neuromyelitis optica (NMO) is an inflammatory disease predominantly affecting the optic nerves and spinal cord. The discovery of NMO-immunoglobulin G (NMO-IgG), targeting aquaporin-4 (AQP4),[1,2] suggested that NMO is a distinct entity with a fundamentally different aetiology from that of multiple sclerosis (MS). NMO-IgG/anti-AQP4 antibodies show high specificity but medium sensitivity for NMO, and vary by ethnicity.[3] The presence of NMO-IgG/anti-AQP4 antibodies has been repeatedly shown to be significantly associated with frequent relapses and severe visual impairment by mono- or multi-variate analyses.[4–6] NMO usually leads to grave disability because of severe tissue destruction; however, several reports have described a benign form of NMO with a long disease term.[7–9] The immunological features of such benign cases of NMO, as well as the pathogenic mechanisms, remain to be elucidated.

The standard method for detecting anti-AQP4 antibodies is a cell-based immunofluorescence assay (IFA) using AQP4-transfected cells. This method is time-consuming

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and dependent on the user's skill, especially the quantification by serial dilution. Reports of quantitative IFA analyses of anti-AQP4 antibodies are relatively few. Takahashi et al.[10] found a significant positive correlation of IFA-determined anti-AQP4 antibody titer with spinal cord lesion length, which has not been replicated. We previously reported a nearly significant negative correlation between NMO-IgG titers and the Progression Index.[5] Jarius et al.[11] described a correlation between anti-AQP4 antibody titer and the occurrence of relapses in NMO patients, while a fraction of NMO patients in their longitudinal study showed a steady rise in anti-AQP4 antibody titer over time without any relapses. The clinical relevance of anti-AQP4 antibody titers is yet to be determined.

Several studies have reported different techniques that could be used for quantification of anti-AQP4 antibodies, such as a flow cytometric assay (FCMA), radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA).[12–14] However, quantitative comparisons of data generated by these different methods have not been performed. Moreover, the anti-AQP4 antibodies tested thus far by IFA mostly belong to the complement-fixing immunoglobulin 1 (IgG1) subclass,[6] and antibodies in other IgG subclasses have not been studied extensively. Therefore, we developed a quantitative FCMA for anti-AQP4 antibody IgG subclass-specific analyses and applied a bridging ELISA for a longitudinal study of anti-AQP4 antibody levels. We quantitatively compared IFA, FCMA and ELISA methods, and characterised the clinical relevance of the titer and IgG subclass of anti-AQP4 antibodies in NMO.

Materials and methods

Subjects

MS was diagnosed according to the revised McDonald criteria,[15] while NMO was diagnosed according to criteria published in 1999,[16] primarily because NMO-IgG/anti-AQP4 antibody status is not included as a diagnostic item. Longitudinally extensive myelitis (LM) was defined as myelitis with longitudinally extensive spinal cord lesions (LESCLs), extending over three or more vertebral segments on MRI. All patients were examined in the Department of Neurology, Kyushu University Hospital, between April 1994 and March 2010. We collected serum samples from 142 MS patients, comprising 119 with relapsing–remitting MS (RRMS), 14 with secondary progressive MS (SPMS) and nine with primary progressive MS (PPMS), 29 NMO patients, 19 patients with either recurrent myelitis (RM) or LM (RM/LM), 57 patients with other inflammatory neurological diseases (OINDs), 29 patients with other non-inflammatory neurological diseases (ONDs), and 28 healthy controls (HCs). To confirm the specificity of the ELISA for anti-AQP4 antibodies, we also studied 41 OND cases (40 with spinocerebellar degeneration and one with cervical spondylotic radiculopathy) and 138 HCs by ELISA alone. In addition, samples from nine patients whose sera were positive for anti-AQP4 antibodies, and from whom samples were taken two or more times during relapse or remission, as defined below, were also included to study a relationship between ELISA values and disease stage. To compare ELISA titers and disease stage, we used the first blood samples collected after the initiation of relapse whenever multiple samples were collected during a relapse phase. All relapse phase sera were taken before administration of immunotherapies, within one month after the initiation of relapse. Sera from patients in the remission phase were taken at least 30 days after the previous relapse and at least 100 days before any subsequent relapse based on a previous report that anti-AQP4 antibody titers increased by 124−294% within the period 48–99 days before relapse.[11] We collected demographic data from the patients with anti-AQP4 antibodies by retrospective review of their medical records, including Expanded Disability Status Scale (EDSS) of Kurtzke scores,[17] Progression Index,[18] annual relapse rate, maximum spinal cord lesion length on MRI, history of LESCLs and acute transverse myelitis (ATM),[19] fulfilment of the Paty[20] and Barkhof[21] criteria, and the presence of serum anti-SS-A and anti-SS-B antibodies at the time of blood sampling. Furthermore, we collected clinical data on the severity of optic neuritis; we regarded ON for which the functional scale was greater than five as severe optic neuritis.[5] All enrolled individuals were Japanese. The protocol was approved by the ethical committee of Kyushu University, and informed consent was obtained from each subject.

Immunofluorescence assay

Anti-AQP4 antibodies were measured using green fluorescent protein (GFP)-labeled AQP4 (M1 isoform) fusion protein-transfected human embryonic kidney (HEK293) cells.[5,6] Fifty microlitres of the serum samples were diluted 1:4 in Dulbecco's modified Eagle's medium and assayed at least twice, with the examiners blinded to the origin of the specimens. Samples that gave a positive result twice were regarded as positive. Positive samples at the standard concentration were titrated with successive doubling dilutions.

Flow cytometric assay

We generated HEK293 cells stably transfected with a construct expressing AQP4 (M1 isoform) fused at its N-terminus to GFP. GFP-AQP4 -transfected and untransfected HEK293 cells were evenly mixed, and 2.5×10^5 mixed cells were suspended in 75 μl of phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA). Twenty-five microlitres of serum was added to

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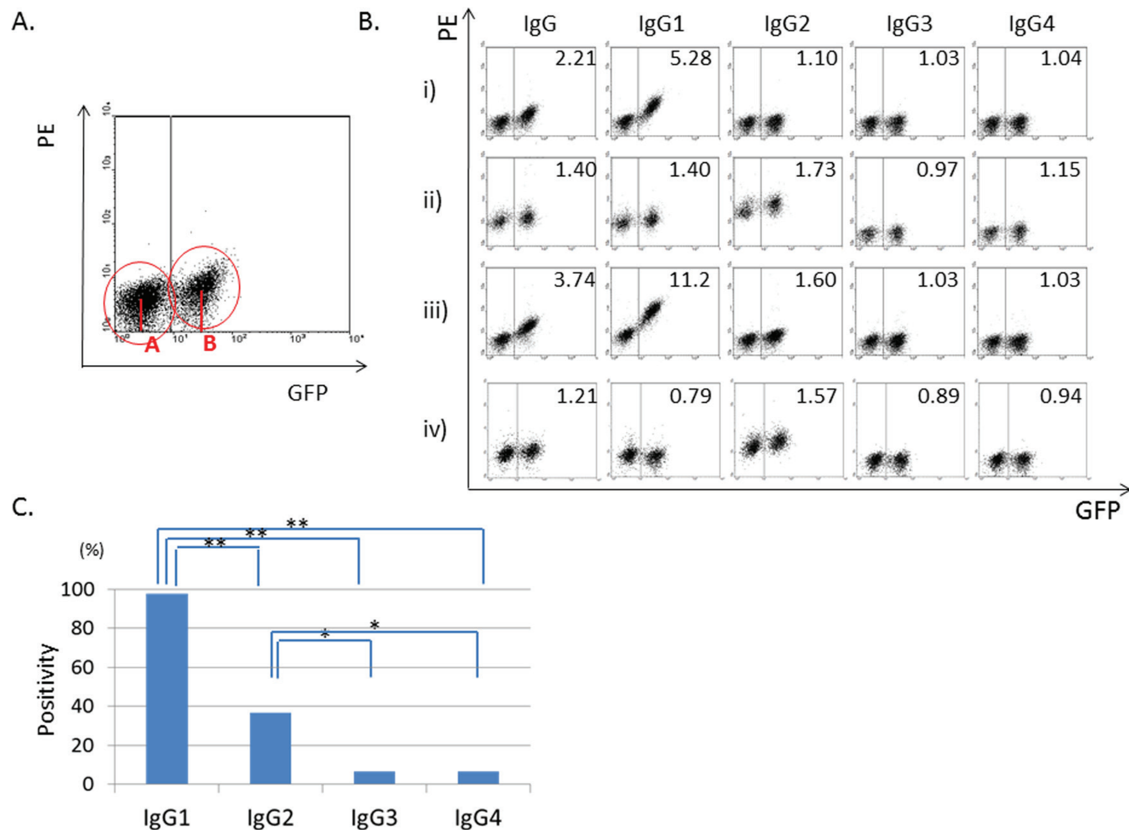

Figure 1. Flow cytometric assay (FCMA) for detecting anti-aquaporin-4 (AQP4) antibodies.
(A) There are two groups of HEK293 cells: one comprising untransfected HEK293 cells (red circle, A), and the other comprising cells stably transfected with GFP-AQP4 (red circle, B). The mean fluorescence intensity (MFI) ratio for PE was calculated by dividing the MFI-PE for group B cells by that for group A cells. If the MFI ratio for immunoglobulin G (IgG) was greater than 1.2, we regarded the result as positive.
(B) Representative analyses of IgG subclass. (i) A sample from a neuromyelitis optica (NMO) case demonstrating positivity for IgG1 subclass anti-AQP4 antibodies. (ii) A sample from another NMO case who had both IgG1 and IgG2 subclasses of anti-AQP4 antibodies. In this case, the IgG2 subclass had a higher MFI ratio than that for IgG1. (iii) A sample from an MS case demonstrating positivity for both the IgG1 and IgG2 subclasses of anti-AQP4 antibodies; the IgG1 subclass was the more prevalent. (iv) A sample from an MS case who was positive for only the IgG2 subclass.
(C) The positivity rate of each IgG subclass of anti-AQP4 antibody among 46 cases in whom anti-AQP4 antibodies were detected by FCMA (**$p^{corr}<0.0001$, *$p^{corr}<0.01$). Serum was diluted 1:4 except in the the subclass analysis, for which the serum was not diluted.
GFP: green fluorescent protein; PE: phycoerythrin

the cell solution for 30 minutes at 4°C. After washing cells twice with 1% BSA-PBS, phycoerythrin (PE)-conjugated mouse anti-human IgG (1:100 dilution in 1% BSA-PBS, 25 µl; Beckman Coulter, Fullerton, CA, USA) was added as the secondary antibody. After a 10-minute incubation at 4°C, the cells were washed again with 1% BSA-PBS and resuspended in 1% paraformaldehyde for two-color flow cytometry using an Epics XL System II (Coulter Electronics, Inc., Hialeah, FL, USA). Ten thousand events per sample were measured. As a negative control, one sample of cell mixture per assay was prepared without serum. We analysed the results using WinMDI version 2.9 software (Scripps Research Institute, La Jolla, CA, USA). The mean fluorescence intensity (MFI) ratio (Figure 1A) was calculated as follows: {MFI-PE (MFI for the secondary antibody tag PE) of the GFP-AQP4-transfected cell group} / {MFI-PE of the untransfected cell group}. Based on preliminary data from 16 HC sera without NMO-IgG (measured by V. A. Lennon, Mayo Clinic, Rochester, MN, USA), we determined the upper normal limit of the FCMA as 1.2, (mean+5 SD). The 28 HC sera samples used for comparison in the assays showed MFI ratios of 0.361–1.096. Serum samples that were positive in the assay were then tested again using PE-conjugated mouse anti-human IgG1, IgG2, IgG3 or IgG4 (1:100 dilution in 1% BSA-PBS, 25 µl; Beckman Coulter). We also conducted subclass analysis of cases with anti-AQP4 antibody detected by means other than FCMA, as well as 38 cases (26 OIND and 12 OND) and 25 controls. In the subclass analyses of anti-AQP4 IgG antibody, we set the upper normal limit as 1.24, considering the slightly higher background of the undiluted HC sera. For analysis of the associations between the MFI ratios for each anti-AQP4 antibody subclass and clinical parameters, cases with both total IgG anti-AQP4 antibody and at least one subclass of anti-AQP4 antibody were used.

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Enzyme-linked immunosorbent assay

Purified preparations of recombinant human AQP4 (RSR Ltd., Cardiff, UK), expressed in insect cells from a human AQP4 cDNA encoding amino acids 1–323, were coated onto ELISA plate wells (Nunc A/S, Roskilde, Denmark) at 15 ng per well as described previously.[22] Purified preparations of recombinant AQP4 were biotinylated using NHS-biotin from Perbio Science (Tattenhall, UK) according to the manufacturer's instructions. In brief, 50 μl aliquots of serum samples together with 25 μl of AQP4-biotin (15 ng) were incubated in AQP4-coated ELISA plate wells at room temperature (RT) for two hours (shaking, 500 shakes per minute). The plate wells were then washed with wash buffer (150 mmol/l NaCl, 20 mmol/l Tris, 1.0 ml/l NP40, pH 7.7) and incubated for 20 minutes with 100 μl of streptavidin peroxidase conjugate. A wash step was then followed by incubation with tetramethylbenzidine (100 μl) for 20 minutes in the dark at RT followed by addition of 0.25 mol/L H_2SO_4 (100 μl) and measurement of the optical density (OD) at 405 and 450 nm. Anti-AQP4 antibody concentration in the test samples was calculated from a calibration curve prepared from a reference preparation for anti-AQP4 antibodies at different dilutions (RSR Ltd.). ELISAs were carried out by JF, SC, JF, and BRS (RSR Ltd.) in an examiner-blinded fashion.

Statistical analysis

Correlations among the anti-AQP4 antibody titers determined by the three different methods, and between the anti-AQP4 antibody levels and clinical parameters, were analysed by Spearman's rank correlation test. To calculate the sensitivity and specificity, we considered that all cases who met the 1999 diagnostic criteria for NMO were positive for anti-AQP4 antibodies, while all OIND cases, all OND cases, all HCs and all MS cases who did not have LESCLs were negative for the antibodies. We also provided 95% CIs for both sensitivity and specificity. To analyze the differences in the proportions of each subclass among participants with anti-AQP4 antibody by FCMA, the χ^2 test or Fisher test and the Bonferroni–Dunn correction (p^{corr}) were used. Comparisons of anti-AQP4 antibody titers by ELISA between relapse and remission phases in paired samples from the same patient were conducted using the Wilcoxon signed rank test. To analyze the association between the level of anti-AQP4 antibody determined by each method and clinical parameters, adjustment of p values for multiple comparisons was conducted using the Benjamini-Hochberg method in R version 2.13.0 software (The R Foundation for Statistical Computing, Vienna, Austria). All analyses other than multiple corrections were performed using JMP 7.0.1 software (SAS Institute, Cary, NC, USA). The threshold for statistical significance was set at $p<0.05$ (two-tailed).

Results

Detection of anti-AQP4 antibodies by FCMA

When samples were plotted according to their GFP and PE fluorescence intensities on FCMA, the GFP-AQP4-transfected and untransfected cells were clearly separable (Figure 1), with positive sera reacting against the former cells but not the latter.

Quantification of anti-AQP4 antibodies

Significant positive correlations were observed between IFA-determined anti-AQP4 antibody titers and ELISA values, between IFA-determined titers and FCMA MFI ratios, and between ELISA values and FCMA MFI ratios (Figure 2).

Detection of anti-AQP4 antibodies

Among the 29 NMO patients, 12 (41.4%) were positive for anti-AQP4 antibodies by IFA, 15 (51.7%) were positive by FCMA and 14 (48.3%) were positive by ELISA (Table 1). Among the 142 MS patients, anti-AQP4 antibodies were detected in 17 patients (12.0%) by IFA, 25 patients (17.6%) by FCMA and 20 patients (14.1%) by ELISA (Table 2). Twenty-four RRMS cases (20.2%) were positive for anti-AQP4 antibody by at least one of the three methods, while one SPMS case (7.1%) with LESCLs was positive for the antibody. Among the nine PPMS patients, one was positive by FCMA alone; this patient had a LESCL with the first available brain MRI fulfilling the Barkhof criteria. Although the antibody positivity rates were smaller in the SPMS and PPMS groups compared with those in the RRMS group, there was no significant difference in the frequency of patients with anti-AQP4 antibody detected by at least one method among the three groups (p=0.6611). Among the 19 RM/LM patients, anti-AQP4 antibodies were detected in six (31.6%, 1/9 with RM and 5/10 with LM) by IFA and FCMA and seven (36.8%, 2/9 with RM and 5/10 with LM) by ELISA.

Sensitivity and specificity of IFA, FCMA and ELISA

All samples from the 57 OIND patients, the 29 OND patients and the 28 HCs were negative for anti-AQP4 antibodies by IFA, FCMA and ELISA (Table 1). We extended the ELISA assay to an additional 138 HCs and 41 OND patients, and we found that none was positive for anti-AQP4 antibodies (0.238±0.469 U/ml for HC and 0.304±0.278 U/ml for OND). In 95 MS cases without LESCLs, six cases were positive for the antibody by IFA and FCMA and seven cases were positive by ELISA. Finally, 50 cases (16.4%) out of 304 participants were positive for anti-AQP4 antibody by at least one method. Among these, 35 (70.0%) were positive by IFA, 46 (92.0%) were

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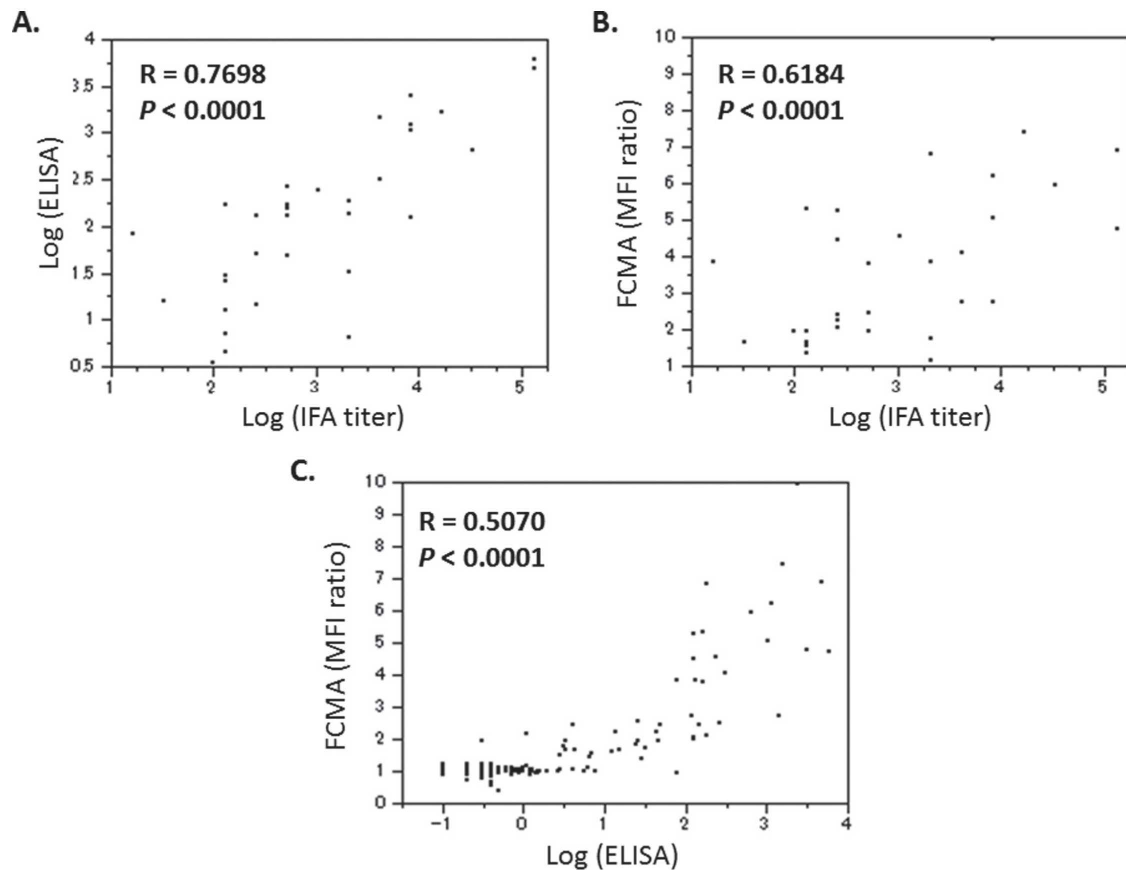

Figure 2. Correlations among the titers of anti-aquaporin-4 (anti-AQP4) antibodies determined by cell-based immunofluorescence assay (IFA), flow cytometric assay (FCMA) and enzyme-linked immunosorbent assay (ELISA).
(A) Correlation between the logarithm of the IFA-determined titer and the logarithm of the ELISA value.
(B) Correlation between the logarithm of the IFA-determined titer and the mean fluorescence intensity (MFI) ratio.
(C) Correlation between the logarithm of the ELISA value and the MFI ratio. In the ELISA, anti-AQP4 antibody reference preparations at 160, 75, 30 and 5 U/mL typically showed optical density(OD) values at 450 nm of 3.536, 1.888, 0.854 and 0.177, respectively, and a healthy blood donor pool alone showed an OD at 450 nm of 0.005. When 166 healthy control (HC) sera were tested by ELISA, anti-AQP4 antibody values ranged from 0.0 to 4.9 U/ml (mean 0.18 U/ml); consequently, the limit of detection of anti-AQP4 antibodies by the ELISA assay was determined to be ≥ 5.0 U/ml (based on 100% HC samples).
IF titer: IFA-determined titer

positive by FCMA, and 41 (82.0%) were positive by ELISA.

The sensitivities of the three methods were as follows: 41.4% for IFA; 51.7% for FCMA; and 48.3% for ELISA. The specificities were 97.1%, 97.1%, and 96.7% respectively (Table 1).

Distribution of anti-AQP4 antibodies in MS using the revised NMO criteria

When we applied the revised NMO criteria,[23] including positivity for anti-AQP4 antibody, to MS cases who were positive for anti-AQP4 antibodies by each method, 13 out of 17 cases (76.5%) with the antibody by IFA, 17 out of 25 cases (68.0%) with the antibody by FCMA, and 14 out of 20 cases (70.0%) with the antibody by ELISA fulfilled the revised NMO criteria (Table 2). In total, among the 26 cases whose serum was positive for anti-AQP4 antibodies by at least one method, 17 cases met the revised NMO criteria, while an additional five cases had LESCLs and were regarded as having an NMO spectrum disorder (NMOSD).[24] Among 47 MS cases with LESCLs, 20 (42.6%) cases fulfilled the revised NMO criteria. Among the other 95 MS cases without LESCLs, seven (7.4%) cases were positive for anti-AQP4 antibody by at least one method and three (3.2%) cases met the revised NMO criteria. Among the remaining 92 MS patients who neither met the revised NMO criteria nor had LESCLs, four (4.3%) were positive for anti-AQP4 antibodies (Supplementary Material, Table 1). Among these four patients, one patient (MS3) was positive only by ELISA and could have been a false positive. Another (MS4) showed neither brain MRI lesions fulfilling the Barkhof criteria[21] nor cerebrum-derived symptoms and thus might have had an NMO spectrum disorder. Focusing on MRI brain lesions fulfilling the Barkhof criteria, 58 MS cases met the Barkhof criteria at their latest brain MRI and

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Table 1. Seropositivity for anti-aquaporin-4 (anti-AQP4) antibodies among cases with neurological diseases and healthy controls.

		No.	Age (yrs)	Sex (M:F)	IFA (+) *n* (%)	FCMA (+) *n* (%)	ELISA (+) *n* (%)
Disease Group:	NMO[a]	29	49.3±14.4	3:26	12 (41.4)	15 (51.7)	14 (48.3)
Control Group:	MS W/O LESCLs[b]	95	38.3±11.1	33:62	6 (6.3)	6 (6.3)	7 (7.4)
	OIND[c]	57	46.0±17.7	22:35	0 (0.0)	0 (0.0)	0 (0.0)
	OND[d]	29	54.9±14.6	14:15	0 (0.0)	0 (0.0)	0 (0.0)
	HCs	28	44.7±14.7	13:15	0 (0.0)	0 (0.0)	0 (0.0)
Sensitivity[e] (%) (95% CI, %)					41.4 (25.5−59.3)	51.7 (34.4−68.6)	48.3 (31.4−65.6)
Specificity[f] (%) (95% CI, %)					97.1 (93.9−98.7)	97.1 (93.9−98.7)	96.7 (93.2−98.4)

ELISA: enzyme-linked immunosorbent assay; FCMA: flow cytometric assay; HC: healthy control; IFA: immunofluorescence assay; M:F: male:female; LESCLs: longitudinally extensive spinal cord lesions; MS: multiple sclerosis; NMO: neuromyelitis optica; OIND: other inflammatory neurological diseases; OND: other non-inflammatory neurological diseases; W/O: without.
[a]Cases who fulfilled the 1999 criteria for NMO.
[b]MS W/O LESCLs were diagnosed according to the revised McDonald criteria (2005) and the numbers of patients with relapsing–remitting MS, secondary progressive MS and primary progressive MS were 81, 7, and 7 respectively.
[c]The patients with OIND included 13 with atopic myelitis, eight with parasitic myelitis, seven with Sjögren's syndrome (two with peripheral neuritis, two with myelitis and peripheral neuritis, two with myelitis, and one with cerebellar ataxia), six with encephalitis, six with HTLV-I-associated myelopathy, three with myasthenia gravis, three with sarcoidosis, two with systemic lupus erythematosus (SLE) (encephalopathy), two with Behçet disease, two with Guillain-Barré syndrome and one with each of acute disseminating encephalomyelitis, Churg-Strauss syndrome, meningitis, paraneoplastic syndrome and myelitis of unknown cause.
[d]The OND patients comprised 11 cases with spinocerebellar degeneration, five with amyotrophic lateral sclerosis, three with multiple system atrophy, three with Parkinson's disease, two with normal pressure hydrocephalus and one with each of diffuse Lewy body disease, temporal lobe epilepsy, hereditary spastic paraplegia, cervical spondylotic myelopathy and cardiogenic cerebral infarction.
[e]Sensitivity was calculated by assuming that cases with NMO possess anti-AQP4 antibodies.
[f]Specificity was calculated by assuming that healthy controls, cases with OND or OIND and MS cases without LESCLs do not possess anti-AQP4 antibodies.
One NMO case was positive for anti-AQP4 antibodies by IFA (2048×) and ELISA (6.2 U/ml) but was negative by FCMA (MFI ratio=1.08). Two NMO cases were positive by FCMA only (the mean fluorescence intensity (MFI) ratios were 1.74 and 1.88 respectively) but were negative by IFA and ELISA (3.2 U/ml and 0.3 U/ml respectively). Two other cases with NMO were positive by both FCMA and ELISA (the MFI ratios were 1.33 and 2.05 respectively; the ELISA values were 24.9 U/ml and 180.5 U/ml respectively) but were negative by IFA.

did not meet the revised NMO criteria or have LESCLs. Among them, two (3.4%) cases were positive for anti-AQP4 antibody by at least one method of detection.

IgG subclass analysis by FCMA

Among the 46 patients positive for anti-AQP4 antibodies by FCMA, the IgG1 subclass was detected in all but one RRMS patient who possessed only IgG2 antibodies (Figure 1B). In the 45 cases with IgG1 subclass antibodies, the IgG1 subclass was dominant in all patients, except for one PPMS patient (IgG2 dominant) and one NMO patient (IgG2 dominant) (Table 3). IgG1 was the most frequent subclass (97.8%) and IgG2 was the second most frequent subclass (37.5%) among cases with anti-AQP4 antibody by FCMA. The frequency of IgG1 was significantly higher than that of any other subclass while that of IgG2 was significantly higher than that of IgG3 and IgG4 (Figure 1C). Among the 46 cases with anti-AQP4 antibodies detected by FCMA, the MFI ratios for IgG1 were significantly higher than those for IgG2 anti-AQP4 antibodies (median (range): 8.0 (0.8–28.2) and 1.1 (0.6–2.5), respectively, $p<0.0001$). The MFI ratio for IgG1 anti-AQP4 antibody in cases positive for the subclass of the antibody was 8.3 (1.3–28.2), while that for IgG2 anti-AQP4 antibody in cases positive for that subclass was 1.6 (1.2–2.5) ($p<0.0001$). When we measured positivity of IgG subclasses of antibodies towards AQP4 in four cases with anti-AQP4 antibody detected by methods other than FCMA, one MS patient was positive for the IgG2 subclass while one NMO patient and one RM/LM case were positive for both IgG1 and IgG2 subclasses (Supplementary Material, Table 2). We also assessed IgG subclasses of anti-AQP4 antibody in 63 samples from 26 OIND cases, 12 OND cases and 25 HCs who were all negative for the anti-AQP4 antibody by all of the three methods. The two OIND cases, one patient with Sjögren's syndrome and progressive spinal muscular atrophy (IgG1) and one patient with systemic lupus erythematosus (SLE) and encephalopathy (IgG2), were positive for one subclass, although their antibody levels were very low (Supplementary Material, Table 3).

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Table 2. Seropositivity for anti-aquaporin-4 (anti-AQP4) antibodies among multiple sclerosis (MS) and recurrent myelitis (RM)/longitudinally extensive myelitis (LM) cases with subcategorization of MS cases according to the fulfillment of the revised neuromyelitis optica (NMO) criteria of 2006.

	No.	IFA (+) n (%)	FCMA (+) n (%)	ELISA (+) n (%)
Whole MS[a]	142	17/142 (12.0)	25/142 (17.6)	20/142 (14.1)
NMO 2006 (+)	–	13/22 (59.1)	17/23 (73.9)	14/22 (63.6)
NMO 2006 (-)	–	4/120 (3.3)	8/119 (6.7)	6/120 (5.0)
RRMS	119	17/119 (14.3)	23/119 (19.3)	18/119 (15.1)
NMO 2006 (+)	–	13/21 (61.9)	16/22 (72.7)	13/21 (61.9)
NMO 2006 (–)	–	4/98 (4.1)	7/97 (7.2)	6/98 (6.1)
SPMS	14	0/14 (0.0)	1/14 (7.1)	1/14 (7.1)
NMO 2006 (+)	–	0/1 (0.0)	1/1 (100.0)	1/1 (100.0)
NMO 2006 (–)	–	0/13 (0.0)	0/13 (0.0)	0/13 (0.0)
PPMS	9	0/9 (0.0)	1/9 (11.1)	0/9 (0.0)
NMO 2006 (+)	–	0/0	0/0	0/0
NMO 2006 (–)	–	0/9 (0.0)	1/9 (11.1)	0/9 (0.0)
RM/LM	19	6/19 (31.6)	6/19 (31.6)	7/19 (36.8)

ELISA: enzyme-linked immunosorbent assay; FCMA: flow cytometric assay; IFA: immunofluorescence assay; M:F:male:female; PPMS: primary progressive MS; RRMS: relapsing–remitting MS; SPMS: secondary progressive MS

[a]95 MS cases with longitudinally extensive spinal cord lesions (LESCLs) who are included in the Table 1 are also in this table. All of the MS cases fulfilled the McDonald criteria (2005). Among the 26 MS cases whose sera were positive for the anti-AQP4 antibodies by at least one method, 17 (65.4%) cases met the revised NMO criteria and an additional five cases had LESCLs. Of the remaining four patients, one had a brainstem-spinal form of MS and did not meet the Barkhof criteria and one yielded a very low positive result by ELISA but was negative by FCMA and IFA; overall, two out of the four met the Barkhof criteria; two out of the four had a history of ON and the other two had a history of myelitis (see Supplementary Material, Table 1 for further details).

Seven MS cases were positive for anti-AQP4 antibodies by FCMA and negative by IFA, among whom three cases were also positive for the antibodies by ELISA. One MS case was positive for anti-AQP4 antibodies by IFA (128×) and FCMA (mean fluorescence intensity (MFI) ratio=1.60), while the ELISA results were negative (4.3 U/ml), although the ELISA value was close to the cut-off point. There was one MS patient in whom antibodies were detected by ELISA only (7.5 U/ml). One RM/LM case was positive for the antibody by IFA (100×) and FCMA (MFI ratio=1.91) but negative by ELISA (3.3 U/ml), while there were two RM/LM cases in whom the antibody was detected by ELISA only (78.7 U/ml and 5.6 U/ml, respectively).

Correlations between anti-AQP4 antibody titers and clinical parameters

When we analysed anti-AQP4 antibody-positive patients not receiving corticosteroids, there were no significant correlations between anti-AQP4 antibody titers determined by IFA or by ELISA and any of the clinical parameters examined (Table 4). The MFI ratios for either IgG1 or IgG2 subclass anti-AQP4 antibodies also showed no significant correlation with any parameter examined after correction for multiple comparisons, while the MFI ratio of total IgG to AQP4 showed a significant negative correlation with the Progression Index.

ELISA values did not differ significantly between relapse (within one month after initiation of the relapse and with no corticosteroid treatment, 4.5±4.0 days after relapse initiation, n=28) and remission (606.3±525.1 U/ml at relapse vs 1612.6±3112.4 U/ml in remission; p=0.9530, n=79). Even when only paired samples were compared, the anti-AQP4 antibody values were not significantly different between relapse and remission phases (10 pairs from seven patients, 521.8±477.1 U/ml at relapse vs 713.5±1907.0 U/ml in remission, p=0.1289).

Discussion

This study is the first to investigate the comparative utility of IFA, FCMA and ELISA for quantifying anti-AQP4 antibodies, in conjunction with IgG subclass analysis, and to assess the relationships between the levels of antibodies of each subclass and clinical parameters in a relatively large number of cases. The FCMA had the highest sensitivity, suggesting that preservation of epitope conformation might be critical. The ELISA is based on the ability of autoantibodies to act divalently and form a bridge between AQP4 coated onto ELISA plate wells and AQP4-biotin in a liquid phase using purified recombinant human AQP4 preparations. This double capture approach reduces the capacity of non-specific binding antibodies to be detected, increases the volume of test serum that can be used, and yields good sensitivity and 96.7% specificity. Mader et al. reported that NMO-IgG reacts preferentially with the M23 isoform rather than the M1 isoform.[25] One IFA study using the M23 isoform reported a positivity rate among selected Japanese NMO patients only slightly higher than that in our study.[26] Kalluri et al. recently reported that anti-AQP4 antibody detection by FCMA was not significantly different between

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Table 3. Subclass analysis of anti-aquaporin-4 (anti-AQP4) antibodies by flow cytometric assay (FCMA) among 46 cases with total immunoglobulin G (IgG) anti-AQP4 antibody by FCMA.

	No.	total IgG (+) *n* (%)	Positivity of each subclass, *n* (%)				Main subclass (*n*)[a]	Combinations of IgG subclasses (*n*)
			IgG1	IgG2	IgG3	IgG4		
MS, total	142	25 (17.6)	24 (96.0)	10 (40.0)	3 (12.0)	2 (8.0)	IgG1 (23), IgG2 (2)	1 (14), 1,2 (5), 1,2,3 (3), 1,2,4 (1), 1,4 (1), 2 (1)
NMO 2006[b] (+)	23	17 (73.9)	17 (100.0)	7 (41.2)	3 (17.7)	0 (0.0)	IgG1 (17)	1 (10), 1,2 (4), 1,2,3 (3)
NMO 2006 (–)	119	8 (6.7)	7 (87.5)	4 (37.5)	0 (0.0)	2 (25.0)	IgG1 (6), IgG2 (2)	1 (4), 1,2 (1), 1,2,4 (1), 1,4 (1), 2 (1)
RRMS	119	23 (19.3)	22 (95.7)	9 (39.1)	3 (13.0)	2 (8.7)	IgG1 (22), IgG2 (1)	1 (13), 1,2 (4), 1,2,3 (3), 1,2,4 (1), 1,4 (1), 2 (1)
NMO 2006 (+)	22	16 (72.7)	16 (100.0)	7 (43.8)	3 (18.8)	0 (0.0)	IgG1 (16)	1 (9), 1,2 (4), 1,2,3 (3)
NMO 2006 (–)	97	7 (7.2)	6 (85.7)	2 (28.6)	0 (0.0)	2 (28.6)	IgG1 (6), IgG2 (1)	1 (4), 1,2,4 (1), 1,4 (1), 2 (1)
SPMS	14	1 (7.1)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	IgG1 (1)	1 (1)
NMO 2006 (+)	1	1 (100.0)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	IgG1 (1)	1 (1)
NMO 2006 (–)	13	0 (0.0)	–	–	–	–	–	–
PPMS[c]	9	1 (11.1)	1 (100.0)	1 (100.0)	0 (0.0)	0 (0.0)	IgG2 (1)	1,2 (1)
NMO 2006 (+)	0	–	–	–	–	–	–	–
NMO 2006 (–)	9	1 (11.1)	1 (100.0)	1 (100.0)	0 (0.0)	0 (0.0)	IgG2 (1)	1,2 (1)
NMO 1999[d]	29	15 (51.7)	15 (100.0)	5 (33.3)	0 (0.0)	1 (6.7)	IgG1 (14), IgG2 (1)	1 (9), 1,2 (5), 1,4 (1)
RM/LM	19	6 (31.6)	6 (100.0)	2 (33.3)	0 (0.0)	0 (0.0)	IgG1 (6)	1 (4), 1,2 (2)
Total	304	46 (15.1)	45/46 (97.8)	17/46 (37.0)	3/46 (6.5)	3/46 (6.5)	IgG1 (43), IgG2 (3)	1 (27), 1,2 (12), 1,2,3 (3), 1,2,4 (1), 1,4 (2), 2 (1)

LM: longitudinally extensive myelitis; MS: multiple sclerosis; NMO: neuromyelitis optica; PPMS: primary progressive MS; RM: recurrent myelitis; RRMS: relapsing–remitting MS; SPMS: secondary progressive MS
[a]The number of cases for each main IgG subclass is indicated in brackets. No individuals possessed IgG3 or IgG4 as the main antibody subclass.
[b]The revised NMO criteria in 2006.
[c]The patient showed a chronic progressive myelopathy without optic neuropathy and had both brain lesions fulfilling the Barkhof criteria and LESCLs. The patient was positive for anti-AQP4 antibody by FCMA (mean fluorescence intensity (MFI) ratio for IgG2: 1.5 and IgG1: 1.4) and negative for the antibody by immunofluorescence assay (IFA) and enzyme-linked immunosorbent assay (ELISA) (2.8 U/ml).
[d]Cases who fulfilled NMO criteria in 1999.

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Table 4. The relationship between the serum levels of anti-aquaporin-4 (anti-AQP4) antibodies and clinical parameters among cases not receiving corticosteroids.

Clinical parameters	Anti-AQP4 antibody-positive patients in different assays									
	IFA		FCMA						ELISA	
			IgG		IgG1		IgG2			
	n=14		n=19		n=18		n=8		n=18	
	r	p^{corr}	r	p^{corr}	r	p^{corr}	r	p^{corr}	r	p^{corr}
Age of onset	–0.0465	0.8933	–0.3844	0.4263	–0.1156	0.8651	–0.3952	0.5985	–0.3664	0.4481
Disease duration	0.1218	0.8651	0.4239	0.4263	0.5108	0.3409	0.4910	0.5342	0.2755	0.5751
EDSS score	–0.3053	0.5901	–0.0725	0.8651	0.3119	0.5342	0.4788	0.5342	0.0635	0.8681
Progression Index	–0.2273	0.7522	–0.7401	0.0135*	–0.4316	0.4263	0.1796	0.8651	–0.3624	0.4481
Number of relapses	–0.0944	0.8651	0.0722	0.8651	0.1389	0.8576	0.5714	0.4481	0.2479	0.5985
Number of relapses presenting with optic neuritis	0.0707	0.8681	0.2848	0.5342	0.4165	0.4263	0.7075	0.4263	0.6056	0.1733
Annual relapse rate	–0.4541	0.4263	–0.5018	0.3409	–0.4076	0.4263	0.5000	0.5342	0.0341	0.8933
Maximum spinal cord lesion length	–0.1827	0.8576	–0.0965	0.8651	0.3484	0.5342	–0.2899	0.8576	–0.1193	0.8917
Visual functional scale score at peak[a]	–0.1827	0.7693	–0.1664	0.8576	0.1436	0.8651	0.2052	0.8651	–0.3131	0.5985

EDSS: Expanded Disability Status Scale of Kurtzke; ELISA: enzyme-linked immunosorbent assay; FCMA: flow cytometric assay; IFA: immunofluorescence assay; IFA: immunofluorescence assay; IgG: immunoglobulin G
r =Spearman's rank correlation coefficient; *pcorr* =corrected *p* value
**pcorr* value<0.05 was considered significant.
[a]The visual functional scale score was obtained from cases with a history of optic neuritis.

M1 and M23 isoforms of the antibody.[27] Therefore, we believe that our usage of the M1 isoform did not distort the results. The three methods showed similar trends in their quantitation levels while the differences in positivity rates and clinical correlations may reflect the distinct structures of the antigens employed. Nonetheless, these methods all achieved high specificity and significant correlations with regard to antibody levels. We conclude that FCMA and bridging ELISA, which are user-independent mass analyses, are suitable substitutes for the time-consuming and user-dependent IFA approach.

Although the sensitivities of our FCMA and ELISA were only approximately 50%, positivity rates for NMO-IgG/anti-AQP4 antibodies in NMO cases vary widely (range, 30–70%) among races.[3,28] Our results are comparable to those of Fazio et al. who compared IFA, FCMA and RIA in Italian NMO patients and demonstrated 30–47% sensitivity and 95–100% specificity.[14] In addition, a recent nationwide survey of NMO in France also gave a positivity rate of 48.0% in cases who fulfilled the revised NMO criteria.[29] In our series, only 4.3% of MS patients not meeting the NMO criteria (either 1999 or 2006) or having NMOSDs,[24] or 3.4% of MS patients not meeting NMO criteria or having LESCLs but fulfilling the Barkhof criteria for MRI brain lesions, possessed anti-AQP4 antibodies. NMO-IgG was initially reported to be present in 9% of Caucasian MS patients[1] and 15% of Japanese MS patients.[4] Pittock et al. reported that five of 41 (12.2%) NMO-IgG carriers showed MS-like brain lesions.[30] Since there seems to be a greater overlap in clinical features between MS and NMO among Asians than among Westerners,[3] a long-term observation of cases that are borderline between the two conditions is warranted to elucidate whether the few anti-AQP4 antibody-positive MS-like cases fall into the NMO category.

In the present study, no association of anti-AQP4 antibody levels, as determined by three different methods with any clinical parameter, was found with the exception of a negative association between FCMA total anti-AQP4 IgG levels and the Progression Index. This negative correlation with the Progression Index is in accord with our previous finding.[5] The fact that this negative association was detected only by FCMA could be partly due to the difference in detectable antigen epitopes. Although the Spearman's rank correlation test does not indicate the existence of a causative relationship, it is possible that a subset of patients experience no relapse for a long time, despite high anti-AQP4 antibody titers in their sera, thereby decreasing the Progression Index and annual relapse rate. Otherwise, we found no association of anti-AQP4 antibody titres with any clinical parameter, nor did we find any significant difference in titers between relapse and remission phases. There have been reports of NMO cases with sera harbouring anti-AQP4 antibodies many years before the onset of NMO,[9] and some patients with malignancies have been shown to carry NMO-IgG but have no NMO symptoms,[31] suggesting

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that factors other than NMO-IgG/anti-AQP4 antibodies might be required to initiate CNS inflammation in this condition.

The results of the IgG subclass analyses using our FCMA reveal for the first time the frequent occurrence of IgG2 antibodies against AQP4. Kalluri et al. reported that various subclasses of AQP4-specific antibodies were generated by immunization with full-length AQP4,[32] although the composition and function of the IgG subclasses were different between mice and humans.[33] IgG2 anti-AQP4 antibodies, which have less complement-fixing ability than IgG1 and IgG3 subclasses, may exert distinct effects from IgG1 anti-AQP4 antibody in NMO. However, their possible effects might be small or masked because the IgG1 subclass, which is one of the most complement-activating subclasses, is the major subclass in most IgG2-positive cases.

Finally, in this study, there were unexpected anti-AQP4 antibody-positive cases that should be addressed. One example is a PPMS case presenting with chronic progressive myelopathy without optic neuropathy, who had IgG2 subclass anti-AQP4 antibodies and LESCLs. Anti-AQP4 antibody positivity in the PPMS population is new, and we should confirm whether this finding can be replicated in a much larger PPMS population. There were also two OIND cases who were negative for total IgG anti-AQP4 antibody but gave positive results in the expanded IgG subclass analysis: one had SLE while the other had Sjögren's syndrome. Although these results could be regarded as false positives, they might reflect the presence of low-titer anti-AQP4 antibodies produced by activated humoral immune states.

The importance of early introduction of disease-modifying therapy in patients with MS[34–36] or clinically isolated syndromes[37–39] necessitates differentiation between MS and NMO as early as possible, especially in Asians.[3] FCMA and ELISA are suitable for this purpose, but the clinical relevance of the anti-AQP4 antibody titer and subclass requires cautious interpretation.

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Conflict of interest statement

NI received a postdoctoral fellowship from The Uehara Memorial Foundation. TY, YK, MK, and SY declare that they have no potential conflicts of interest. TM received a grant and payment for manuscript preparation and development of educational presentations from Bayer Schering Pharma, and also received payment for the development of educational presentations from Mitsubishi Tanabe Pharma. JF, SC, JF, and BRS are employees of RSR Ltd. RSR Ltd develops medical diagnostics, including the kits used for measuring AQP4 autoantibodies. JK is an advisory board member for Merck Serono and a consultant for Biogen Idec Japan. He has received payments for lectures from Bayer Schering Pharma, Cosmic Cooperation and Biogen Idec Japan.

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