Establishment of a New Sensitive Assay for Anti-Human Aquaporin-4 Antibody in Neuromyelitis Optica

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Neuromyelitis optica (NMO) is a devastating neurologic disease characterized by severe optic neuritis and transverse myelitis. Recently, its disease-specific serum autoantibody, NMO-IgG, was discovered with indirect immunofluorescence. However, the substrates of the immunofluorescence assay were not human but mouse brain tissues, which could influence the sensitivity and specificity of the antibody. The target antigen of NMO-IgG was recently identified as aquaporin-4 (AQP4) water channel protein, which is mainly expressed in brain and spinal cord. In the present study, we have established human cell lines that stably express human AQP4 and used these cells to detect and titrate anti-AQP4 antibody present in the sera of patients with NMO by immunofluorescence assay. The results were compared with those of the original NMO-IgG assay. We tested the sera from 10 patients with NMO, 10 with MS and five with other neurological disorders. Among the patients with NMO, six were NMO-IgG-positive. However, using the new anti-AQP4 antibody assay, we showed that eight patients with NMO including the six NMO-IgG-positives were positive for anti-AQP4 antibody. The staining pattern of AQP4-expressing cells treated with each serum of these eight NMO patients corresponded to that with a commercially available anti-AQP4 antibody. The antibody titer (maximum serum dilution for positive staining) ranged from 64x to 16,384x. The serum dilution titers were reproducible in blinded studies. In contrast, the patients with MS or other neurological disorders showed negative for anti-AQP4 antibody. Thus, the newly developed anti-AQP4 antibody assay appears to have a higher sensitivity for NMO than the original NMO-IgG assay and is expected to be useful for the diagnosis of NMO.

Keywords: Neuromyelitis optica; NMO-IgG; anti-aquaporin-4 antibody; early diagnosis; antibody titration

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Neuromyelitis optica (NMO) is a devastating immune-mediated neurologic disease that mainly affects the optic nerves and spinal cord. NMO is often relapsing and causes severe visual impairment and transverse myelopathy (Wingerchuk et al. 1999). Whether NMO is a variant of multiple sclerosis (MS) or a distinct entity has long been discussed, but NMO has some features distinct from MS, such as female preponderance, poor prognosis, rare brain MRI abnormalities, longitudinally extensive (≥ three vertebral segments) spinal cord lesions, and the absence of oligoclonal IgG bands (OB) (Wingerchuk et al. 1999). NMO should be distinguished from MS in the early stage of disease, because their optimal treatments are different (Mandler et al. 1998; Keegan et al. 2002; Cree et al. 2005). Thus, laboratory tests useful for the early diagnosis of NMO have been vigorously sought.

A variety of serum autoantibodies including anti-nuclear, anti-thyroid, anti-phospholipid, anti-neutrophil cytoplasmic antibody and SS-A/SS-B have been reported in NMO, but none of them was specific to this disease. In 2004, an NMO-specific serum autoantibody, NMO-IgG, was reported by means of an indirect immunofluorescent (IF) method using mouse brain slices and the sera of North American and Japanese patients (Lennon et al. 2004). NMO-IgG was highly sensitive (58–76%) and specific (94–100%) to NMO, and the NMO-IgG seropositive status is now incorporated in the revised diagnostic criteria of NMO (Lennon et al. 2004; Wingerchuk et al. 2006). However, since the substrates of the NMO-IgG detection assay were not human but mouse brain tissues, there has been a concern that this might affect the sensitivity and specificity of the antibody detection, and in fact there were some patients of typical NMO who were negative for NMO-IgG. The target antigen of NMO-IgG was recently identified as aquaporin-4 (AQP4) water channel protein (Lennon et al. 2005). AQP4 is mainly expressed in the gray matter of the spinal cord, and in the periventricular and periaqueductal areas (Jung et al. 1994; Oshio et al. 2004), and is concentrated at the astroglial foot processes which line the blood brain barrier (BBB) (Vizuete et al. 1999). Consistent with the distribution of AQP4, the brain lesions of NMO were reported to be localized in the periventricular and periaqueductal areas (Misu et al. 2005; Nakashima et al. 2006; Pittock et al. 2006). Moreover, we most recently found the loss of AQP4 in the active perivascular lesions of a typical case of NMO (Misu et al. 2006). These findings strongly suggest that anti-AQP4 antibody not only serves as a disease-specific biomarker, but also plays a crucial pathogenetic role in NMO. However, unlike NMO-IgG assays, the anti-AQP4 antibody assay has not been used clinically.

In the present study, we have established human cell lines that stably express human AQP4 and used these cells to detect and titrate anti-AQP4 antibody present in the sera of patients with NMO by immunofluorescence assay. The results were compared with those of the original NMO-IgG assay.

**MATERIALS AND METHODS**

**Patients and sera**

We analyzed 20 clinically ascertained Japanese patients (10 with NMO and 10 with MS) in the present study. All the patients were diagnosed as having NMO or MS by the criteria shown below. NMO was defined as cases fulfilling all items of the revised criteria of NMO (Wingerchuk et al. 2006) except for the NMO-IgG-seropositive status. The criteria are as follows: 1) optic neuritis, 2) acute myelitis, 3) contiguous spinal cord MRI lesions extending over 3 vertebral segments, 4) onset brain MRI not meeting Paty’s diagnostic criteria of brain MRI for MS (Paty et al. 1988). The demographic data of NMO were as follows: age (median 51 years old [range 23-71]), male/female: 0/10, onset age (37 years old [13-58]), Expanded disability status scale (EDSS) (6.0 [3.5-6.5]). All of 10 cases had documented relapses and none was OB positive. MS was defined as cases fulfilling the revised diagnostic criteria of MS by the international panel (Polman et al. 2005). Their demographic data were as follows: age (36 years old [28-45]), male/female: 0/10, onset age (26 [20-45]), EDSS (3.0 [1.0-8.0]). Five of the patients with MS were OB positive. We also tested 5 sera of other neurological disorders (two with Hashimoto encephalopathy, two with Neuro-Bechet and one with autoimmune encephalopathy; male/female: 4/1), though NMO-IgG were not tested.
in those patients. All of these 25 sera were obtained in 2002-2005 and cryopreserved at -80°C until use in this study.

The study was approved by the institutional review board of the Tohoku University School of Medicine, Sendai, Japan. Patients gave spoken consent for the study.

**NMO-IgG assay**

The serum samples were assayed for NMO-IgG by the reported IF technique (Lennon et al. 2004) at the Mayo Clinic Neuroimmunology Laboratory (Rochester, MN, USA) in 2003-2005 and were judged positive or negative without any knowledge of the patients’ clinical information. Among the 10 samples of NMO, six had been shown to be positive for NMO-IgG. The titers of NMO-IgG in those six patients were not available.

**Anti-human AQP4 antibody assay**

Human AQP4 cDNA was amplified from a Quick Clone cDNA library (adult human cerebellum, BD Biosciences Clontech Laboratories, Mountain View, CA, USA) and cloned into a pTargetTM Mammalian Expression System (Promega, Road-Madison, WI, USA). Human embryonic kidney cells (HEK-293) were stably transfected with pTargetTM with or without AQP4 using Fugene 6 transfection reagent according to the manufacturer’s protocol (Roche Diagnostics, Indianapolis, IN, USA). Stable cell lines were selected using 0.6 mg/ml geneticin (Promega). After the transfection with AQP4 cDNA, about 30-40% of the HEK-293 cells were stained positive for AQP4 with IF as described below, that is, those cells were a mixture of the ones expressing AQP4 and those without AQP4 expression. Meanwhile, the cells transfected with the vector alone were consistently stained negative for AQP4.

We tested all serum samples, which were identical to the ones previously assayed for NMO-IgG, by an IF assay using these two cell lines (with or without AQP4). The procedures were as follows. The serum was diluted at 4× and the HEK-293 cells transfected with AQP4 (the mixture of cells expressing AQP4 and those without AQP4 expression) and those with the vector alone, which were separately cultured on the same plate, were incubated with the diluted serum for 1 hr, washed in phosphate buffered saline (PBS), incubated with fluorescein-conjugated goat anti-human IgG (MP Biomedicals, Aurora, OH, USA) for 30 min and washed in PBS. Then, the cells were fixed in 95% ethanol for 5 min and mounted in the prolonged antifade mounting media Permafluor (Beckman Coulter, Fullerton, CA, USA). When double staining was needed, after fixation with 95% ethanol the cells were incubated with rabbit anti-human AQP4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1h, washed in PBS, incubated with rhodamin-conjugated goat anti-rabbit IgG (Chemicon, Temecula, CA, USA) for 30 min, washed in PBS and mounted in Permafluor. Images were captured using a confocal microscope Fluoview (Olympus, Tokyo) and scored positive or negative by comparing the staining of the cells with AQP4 with those without AQP4. The cut-off was 4×, and antibody-positive serum samples were titrated in serial two-fold dilutions to ascertain the maximum dilution for positive staining.

**RESULTS**

**Anti-AQP4 antibody in NMO and MS**

HEK 293 cells transfected with human AQP4 cDNA were stably stained with commercially available anti-human AQP4 antibodies and the staining was mainly localized on the cell surface (Fig. 1). The staining colocalized with anti-AQP4 staining as shown in the confocal microscopic analysis (Fig. 1) was judged positive. Eight out of 10 sera from the NMO patients were positive. Among those 8 anti-AQP4 antibody-positive serum samples, the antibody titer ranged from 64× to 1,6384× (Fig. 2). The serum dilution titers were confirmed in blinded studies.

On the other hand, the AQP4-expressing cells were not stained with the two other sera of NMO patients or any sera of MS and other neurological disorders, and these were scored negative. No positive staining was observed on the cells without AQP4 cDNA by anti-AQP4 antibody or any serum samples of the 25 patients.

**Comparison of anti-AQP4 antibody status with NMO-IgG status**

Six out of the 8 anti-AQP4 antibody-positive sera of NMO were also positive for NMO-IgG. However, two of the anti-AQP4 antibody-positive sera from NMO were negative in the NMO-IgG assay. The anti-AQP4 antibody titers of the two NMO-IgG-negative sera were 2,048× and 64×, respectively (Fig. 2). Both patients had typical NMO (Patient with the titer of 2,048×; a 50 year-old woman; onset age 39; exacerbations, two
optic neuritis and five myelitis; EDSS 3.5; spinal cord MRI lesions, C6-T8; brain MRI, negative; OB negative) (Patient with the titer of 64×; 71 year-old woman; onset age, 57; exacerbations, three optic neuritis and seven myelitis; EDSS 6.0; spinal cord MRI lesions, C1-4 and T7-9; brain MRI, negative; OB negative). The other 12 sera, two with NMO and 10 with MS, were all negative, which was in agreement with the results in the anti-AQP4 antibody assay. Among the two patients with NMO who tested negative for anti-AQP4 antibody, one had typical NMO (42 year-old woman; onset age, 35; exacerbations, four optic neuritis and nine myelitis; EDSS 6.0; spinal cord MRI lesions, C1-7 and T2-3; brain MRI, negative; OB negative), but the other had a persistently ring-enhanced cervical cord lesion (58 year-old woman; onset age, 49; exacerbations, three

Fig. 1. Indirect immunofluorescence assay of AQP4(−) and (+) cells stained with the serum of a patient with NMO. A: HEK 293 transfected with the AQP4(−) vector, stained with the serum of an NMO patient and fluorescein-conjugated goat anti-human IgG antibody. No signal was observed. B–F: HEK 293 transfected with the AQP4(+) vector. B: Stained with the same serum as the one used in Fig. 1A and fluorescein-conjugated goat anti-human IgG. The cell surface was mainly stained positive. C: Stained with rabbit anti-human AQP4 antibody and rhodamin-conjugated goat anti-rabbit IgG antibody. A similar staining pattern was seen. D: The merging of B and C. The staining colocalized with each other. E: Background cell structures. F: The merging of D and E. The cell surface was definitely stained positive.
optic neuritis and three myelitis; EDSS 8.0; spinal cord MRI lesions, C4-T2; brain MRI, non-specific white matter lesions; OB negative).

**DISCUSSION**

This is the first anti-human AQP4 antibody titration study in the sera of NMO using AQP4-transfected cells, and we also compared the anti-AQP4 antibody status with the NMO-IgG status. As a result, we found that all NMO-IgG-positive sera tested positive for anti-AQP4 antibody, which is consistent with a previous report that the target antigen of NMO-IgG is AQP4 (Lennon et al. 2005). In addition, two sera from NMO-IgG-negative patients tested positive for anti-AQP4 antibody in our assay. Conversely, none of the MS sera was positive for this antibody. Since the anti-AQP4 antibody titers were stable in blinded studies, this method is expected to be useful for comparative assessment of multiple samples.

The fact that two sera from NMO-IgG-negative patients were anti-AQP4 antibody-positive is quite interesting. Those two patients had clinically definite NMO, and one of them showed a relatively high anti-AQP4 antibody titer (2,048×) while the titer was 64× in the other patient. We previously reported a higher rate of permanent complete blindness (no light perception) of at least one eye in NMO-IgG-positive cases than in NMO-IgG-negative cases in Japanese optic-spinal multiple sclerosis (Nakashima et al. 2006) which is essentially equivalent to relapsing NMO (Misu et al. 2002). The two, anti-AQP4 positive, NMO-IgG negative patients had poor visual acuity but were not blind despite recurrent optic neuritis. The discrepancy in the antibody status is probably due not merely to a difference in the antibody detection sensitivity in the two methods, but also to differences between human and mouse AQP4 antigens used as the substrate in the assays. In fact, mouse AQP4 is highly homologous to human AQP4 (95% homology, according to the BLAST search), but there are some differences of amino acid sequences (according to the BLAST search) in the extracellular domains (Hiroaki et al. 2006) between human and mouse AQP4. These might be critically important for the binding of anti-AQP4 antibodies of some patients, although we know nothing about the epitope spectrum of anti-AQP4 antibodies at this point. Further studies are needed to determine if the anti-human AQP4 antibody assay is more sensitive and specific than the NMO-IgG assay due to such an antigen specificity. AQP4 is a water channel protein densely expressed on the cell surface of astroglial foot processes, and the staining pattern of AQP4 with the patients’ sera in our study indicates AQP4 are also expressed on the surface of transfected cells, suggesting that the AQP4-transfected cells are useful to assay anti-AQP4 antibody binding to the extracellular domains of AQP4.

Another advantage of this antibody assay is its simplicity. In this assay, preconditioning of serum such as preabsorption in liver powder is unnecessary. No staining was observed in cells without transfected AQP4 and, practically, we were able to judge them to be either positive or negative patients were anti-AQP4 antibody-positive.
negative without merging the staining of the sera and the commercially available anti-AQP4 antibody. Unlike brain tissues, cell lines can be easily prepared, which is another merit of this assay.

In the present study, two patients with NMO were negative for both anti-AQP4 antibody and NMO-IgG despite fulfilling the diagnostic criteria of NMO. Their sera were obtained in the remission phase. Although one of them (42-year-old woman) had typical NMO, the other one (58-year-old woman) had a persistently ring-enhanced cervical cord lesion throughout the course of the disease, which is not commonly seen in NMO. At present, the reason why those patients were negative for anti-AQP4 antibody is unknown, but there may be at least a few possibilities for the seronegative status in NMO; 1) The antibody titer might be below the cut-off level because the disease is inactive during remission. 2) Some patients with typical NMO and those with atypical features might be truly negative for anti-AQP4 antibody, and the underlying immunological mechanisms are different in such patients. Larger-scale studies are needed to address this issue.

In conclusion, we established a sensitive anti-human AQP4 antibody titration assay which could have higher sensitivity than the original NMO-IgG assay with mouse brain slices. Although larger scale studies need to be done to determine the sensitivities and specificities of anti-AQP4 antibody in NMO and its high-risk syndrome (cases with either recurrent optic neuritis or longitudinally extensive myelitis alone), our anti-human AQP4 antibody assay should be a powerful tool for the early diagnosis of NMO which is imperative for early therapeutic interventions.

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References


Polman, C.H., Reingold, S.C., Edan, G., Filippi, M., Hartung,

