Aquaporin-4 Antibodies in Neuromyelitis Optica and Longitudinally Extensive Transverse Myelitis

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Background: There is increasing recognition of antibody-mediated immunotherapy-responsive neurologic diseases and a need for appropriate immunoassays.

Objectives: To develop a clinically applicable quantitative assay to detect the presence of aquaporin-4 (AQP4) antibodies in patients with neuromyelitis optica and to characterize the anti-AQP4 antibodies.

Design: We compared a simple new quantitative fluorescence immunoprecipitation assay (FIPA) with both indirect immunofluorescence and an AQP4-transfected cell-based assay, both previously described. We used the cell-based assay to characterize the antibodies for their immunoglobulin class, IgG subclass, and ability to induce complement C3b deposition in vitro.

Setting: United Kingdom and Germany.

Participants: Serum samples from patients with neuromyelitis optica (n=25) or longitudinally extensive transverse myelitis (n=11) and from relevant controls (n=78) were studied.

Results: We found antibodies to AQP4 in 19 of 25 patients with neuromyelitis optica (76%) using FIPA, in 20 of 25 patients with neuromyelitis optica (80%) using the cell-based assay, and in 6 of 11 patients with longitudinally extensive transverse myelitis (55%) with both assays; these assays were more sensitive than indirect immunofluorescence and 100% specific. The antibodies bound to extracellular epitope(s) of AQP4, were predominantly IgG1, and strongly induced C3b deposition.

Conclusions: Aquaporin-4 is a major antigen in neuromyelitis optica, and antibodies can be detected in more than 75% of patients. Further studies on larger samples will show whether this novel FIPA is suitable for clinical use. The IgG1 antibodies bind to AQP4 on the cell surface and can initiate complement deposition. These approaches will be useful for investigation of other antibody-mediated diseases.

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sults with the indirect immunofluorescence and cell-based methods and describe the characteristics of the antibodies and their pathogenic potential. Some of these data have been previously reported in abstract form.12

<table>
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<tr>
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<th>Age at Sampling, Median (Range), y</th>
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<td>11:10 (4 unknown)</td>
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<tr>
<td>LEMT (n=11)</td>
<td>3:8</td>
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<td>MS (n=38)</td>
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<td>40.3 (21-74)</td>
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Abbreviations: LETM, longitudinally extensive transverse myelitis; MS, multiple sclerosis; NMO, neuromyelitis optica.

**METHODS**

**PATIENTS**

We studied the earliest serum or plasma samples available from 25 unselected patients from the United Kingdom and Germany with an eventual diagnosis of NMO (defined according to the criteria of Wingerchuk et al9 but discounting the criteria of NMO IgG or AQP4 assay status), 11 patients with longitudinally extensive transverse myelitis (LEMT) who did not fulfill the clinical criteria for NMO, 38 patients with MS (defined according to the criteria of McDonald et al13; 27 with relapsing-remitting MS, 6 with secondary progressive MS, and 5 with primary progressive MS), 26 controls with other autoimmune neurologic disease (AIND) derived from routine referrals, and 14 healthy controls (HCs). Many serum samples were included in our short report using indirect immunofluorescence.14 Table 1 summarizes the patients' demographic and clinical characteristics.

### INDIRECT IMMUNOFLUORESCENCE ASSAY

The NMO IgG was detected by indirect immunofluorescence on 10-µm adult mouse cerebellum cryosections as previously described.14 Sections were classified as positive if they exhibited the typical NMO immunofluorescence pattern.5 For IgM and IgG subclass analysis, goat anti–human IgG was replaced with antibodies to IgG1, IgG2, IgG3, IgG4, or IgM (1:100; The Binding Site, Birmingham, England) and then Alexa Fluor 488–conjugated donkey anti–sheep IgG (1:1000; Invitrogen Ltd, Paisley, England) was added as a secondary antibody. Slides were mounted with standard fluorescent mounting media (DakoCytomation, Cambridge, England) that contained diamidino-2-phenylindole (DAPI) (1:1000).

### SUBCLONING OF HUMAN AQP4

Human AQP4 complementary DNA was obtained (IMAGE clone 4717755; Geneservice Ltd, Cambridge, England). Both AQP4 isoforms were cloned into plasmid EGFP-C3 (Clontech, Saint-Germain-en-Laye, France) using the reverse primer 5'-GCATCCGGGTTCACTGAAAGACAAATACCTTCCGAG and either 5'-GTCACTCGAGATGAGTACGACAGGCCAAGCAGCAAAG or 5'-GTCACTGGAGATGTTGGCTTCAAGGGGTTGCTG to yield plasmid EGFP-AQP4-M1 or 5'-GTCACTCGAGATGTTGGCTTCAAGGGGTTGCTG to yield plasmid EGFP-AQP4-M23.

### CELL-BASED ASSAY AND CHARACTERIZATION OF ANTIBODIES

The HEK 293 cells transfected with both EGFP-tagged AQP4 isoforms using standard polyethyleneimine transfection. Forty hours after transfection with EGFP-AQP4-M1, EGFP-AQP4-M23, or EGFP alone (mock transfection), coverslips were rinsed gently with Dulbecco-modified Eagle medium with 20mM HEPES buffer (4-[2-hydroxyethyl]-1-piperazinethanesulfonic acid) and incubated with patient or control serum samples (1:20) in 250 µL of buffer with 1% bovine serum albumin for 1 hour at room temperature (RT), washed 3 times with buffer, and fixed immediately with 3% formaldehyde in phosphate-buffered saline for 15 minutes at RT. Cells were then rinsed twice and incubated: (1) for the detection of IgG or IgM antibodies, with goat anti–human IgG or goat anti–human IgM Alexa Fluor 568–conjugated secondary antibody (Invitrogen–Molecular Probes, Paisley) at 1:750 in buffer with 1% bovine serum albumin for 45 minutes at RT; and (2) for the identification of IgG subclasses, with mouse anti–human IgG1 or mouse anti–human IgG4 (The Binding Site) at 1:30 in buffer with 1% bovine serum albumin for 1 hour at RT, washed 3 times, and then labeled with goat antio mouse isotype-specific IgG Alexa Fluor 568–conjugated antibody (Invitrogen–Molecular Probes). After antibody labeling, cells were then washed 4 times in phosphate-buffered saline, nuclei were counterstained with DAPI (1:1000), and coverslips were mounted as described herein.

### DETECTION OF C3b DEPOSITION ON AQP4-EXPRESSING CELLS OR MOUSE BRAIN SECTIONS

The HEK 293 cells transfected with AQP4 were incubated with heat-inactivated serum samples (30 minutes at 57°C) from AQP4 antibody–positive patients or controls at 1:20 dilution for 30 minutes at 37°C and then washed briefly. Fresh frozen serum from a healthy donor was used as a source of complement after incubation with HEK 293 cells on ice for 35 minutes to reduce nonspecific complement activation. The preabsorbed serum was applied to the AQP4 antibody–treated cells at 1:20 dilution for 30 minutes at 37°C. The coverslips were fixed in 3% formaldehyde, incubated with a polyclonal rabbit anti–human C3c antibody, which detects C3b (1:500, 30 minutes at 4°C; DakoCytomation, Glostrup, Denmark), or a rabbit isotype control (provided ready for use; Zymed Laboratories, South San Francisco, California); and then incubated with Alexa Fluor 568 goat anti–rabbit IgG antibody for 45 minutes (1:750; Invitrogen–Molecular Probes). All stages were performed with specimens on ice. Coverslips were mounted as described herein using standard fluorescence mounting media (Dako Cytomation) containing DAPI (1:1000). Mouse brain sections were immunostained for C3b in a similar fashion, except that the HEK

### INDIVIDUAL PATIENTS

Table 1. Summary of Clinical Features of Patients

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293 cell preabsorption was not required, and the Alexa Fluor goat anti–rabbit IgG antibody was applied at 1:325.

**DETECTION OF IMMUNOFLUORESCENCE**

Immunofluorescence slides or coverslips were stored, light protected, at 4°C and imaged on a Zeiss fluorescence microscope (Carl Zeiss Ltd, Welwyn Garden City, England) with a MacProbe v4.3 digital image system (Perceptive Scientific Instruments Inc, Chester, England). All photographs were taken under similar conditions. Slides were coded and scored for the intensity of surface immunofluorescence and colocalization with EGFP-AQP4 by 3 of us (P.W., M.I.L., and R.G.). The binding was scored as follows: 0 to 0.5, none or slight labeling without colocalization; 1, weak labeling; 2, moderate labeling; 3, strong labeling; and 4, very strong labeling. The final score was the median of 3 independent readings (variance <1). Sample scores of 1 or more were classified as positive on the basis of control results.

**FLUORESCENCE IMMUNOPRECIPITATION ASSAY**

Flasks of EGFP-AQP4–transfected HEK 293 cells were lysed by incubation for 1 hour at 4°C in 3 mL of extraction buffer (10mM TRIS, 100mM sodium chloride, 1mM EDTA, 1% Triton X-100).

Figure 1. Neuromyelitis optica (NMO) immunoglobulin-binding pattern and determination of IgG subclass and complement activation in NMO serum samples. A, Fluorescin isothiocyanate–labeled anti–human IgG antibody highlights the typical NMO staining pattern around brain microvessels and along the pial layer (original magnification ×200). B, The antibodies were predominantly IgG1. C, After exposure to serum and a fresh source of complement, complement deposition was detected by rabbit anti-C3c and anti–rabbit IgG (red), demonstrating colocalization of NMO immunoglobulin binding (green) and complement C3b deposition.

Figure 2. Aquaporin-4 (AQP4) antibodies measured by the cell-based assay. A, Antibodies binding to AQP4-expressing cells were detected by anti–human immunoglobulin (red) and results scored (the “Cell-Based Assay and Characteristics of Antibodies” subsection in the “Methods” section provides details) (original magnification ×200). B, Scatterplot of scores of each serum sample; 1 or more was considered positive on the basis of the healthy control (HC) group. C, A total of 8 of 15 neuromyelitis optica (NMO) or longitudinally extensive transverse myelitis (LETM) serum samples that were negative by indirect immunofluorescence (IIF) were positive by the cell-binding assay, showing it to be more sensitive ($P < .001$). In B and C, the horizontal line represents the cutoff above which the results are considered positive. The median values are shown in each column. Only results from 10 LETM and 23 NMO serum samples are shown herein. AIND indicates autoimmune neurologic disease; EGFP, enhanced green fluorescent protein; and MS, multiple sclerosis.
Table 2. Summary of Results of the Aquaporin-4 Antibody Assays

<table>
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<th>Diagnosis</th>
<th>No. (%) of Study Participants</th>
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<tr>
<td></td>
<td>In-house Indirect Immunofluorescence&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>NMO (n=25)</td>
<td>14 (58)&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>LETM (n=11)</td>
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<tr>
<td>MS (n=38)</td>
<td>0</td>
</tr>
<tr>
<td>AIND (n=26)</td>
<td>1 (4)</td>
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<tr>
<td>HCs (n=14)</td>
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Abbreviations: AIND, autoimmune neurologic disease; FIPA, fluorescence-based immunoprecipitation assay; HCs, healthy controls; LETM, longitudinally extensive transverse myelitis; MS, multiple sclerosis; NMO, neuromyelitis optica.

<sup>a</sup>The indirect immunofluorescence assay was first established<sup>14</sup> using 3 NMO serum samples (of 8) that were positive for NMO IgG testing by the Mayo Clinic, Rochester, Minnesota.

<sup>b</sup>No serum was available for this assay in 1 patient in each of these groups (for NMO, the denominator was 24; and for LETM, the denominator was 10).

RESULTS

AQP4 ANTIBODIES MEASURED BY INDIRECT IMMUNOFLUORESCENCE AND CELL-BASED ASSAY

Three serum samples previously found positive for NMO IgG at the Mayo Clinic, Rochester, Minnesota, were used to help establish our immunofluorescence assay.<sup>24</sup> Testing all but 1 of our 36 patients with NMO or LETM by this method, we found positive results in 14 of 24 patients with NMO (58%) and in 5 of 10 patients with LETM (50%), with 1 positive serum sample from among 26 AIND controls (+%) but 0 in 38 patients with MS (Figure 1A) or 14 HCs. When tested for IgG subclass, only IgG1 was detected (Figure 1B), and in the presence of 3 NMO IgG-positive serum samples and fresh human serum as a source of complement, C3b deposition was clearly found (Figure 1C), with a distribution highly similar to that of the IgG or IgG1 antibodies.

To confirm that the antibodies bind to extracellular epitopes on AQP4, we expressed AQP4 in HEK 293 cells and used immunofluorescence to detect binding of serum antibodies. Because the EGFP-AQP4 was synthesized in the cytoplasm, green fluorescence was seen both within the cell and at the cell surface. By contrast, patients’ IgG, indicated by red fluorescence, was only detected on the cell surface (Figure 2A). The median scores for each serum sample are shown in Figure 2B. No binding was detectable in HC, AIND, or MS serum samples. The results were positive (≥1.0) in 20 of 25 NMO serum samples (80%) and in 6 of 11 LETM serum samples (55%) (Table 2). More positive results were found with the cell-based assay than with immunofluorescence, and the scores were higher in the immunofluorescence-positive serum samples than in those that were negative (Figure 2C).

CHARACTERIZATION OF THE AQP4 ANTIBODIES USING THE CELL-BASED ASSAY

All positive serum samples tested, including those with both high and low AQP4 antibody reactivity, were strongly IgG1 subclass, as shown for 2 highly positive samples in Figure 3A, and weaker for IgG4 and IgM (Figure 3B). Moreover, 9 of 10 AQP4 antibody-positive serum samples showed strong complement C3b deposition on the cell membrane (Figure 4A and B), which correlated weakly (r² = 0.4, P = .05) with IgG1 AQP4 antibodies (Figure 4C).

AQP4 ANTIBODIES MEASURED BY FIPA

To establish a quantitative and objective assay, we used immunoprecipitation. The EGFP-AQP4, extracted in detergent from the cells, demonstrated a sedimentation coefficient of 7.4, corresponding to that predicted for an EGFP-AQP4 tetramer (Figure 5A). Serum samples that were positive for AQP4 antibodies immunoprecipitated more EGFP-AQP4 with increasing concentrations of EGFP-AQP4 (Figure 5B), suggesting relatively low overall affinity. Serum samples that immunoprecipitated EGFP-AQP4 did not immunoprecipitate EGFP alone or other EGFP-tagged antigens (P.W., unpublished data, 2007). For testing all serum samples, we used 7500 to 9000 fluorescence units (FUs) in a volume of 250 µL, corresponding to 150nM tetrameric EGFP-AQP4 (based on the FUs detected per EGFP) (Figure 5A, insert). A total of 25 µL of the NMO and LETM serum sample immunoprecipitated between 10 and 1600 FUs, corresponding to 2.5 to 400nM of EGFP-AQP4 tetramers per liter of serum. We chose 25 FUs (equivalent to 6.25nM) as a conservative cutoff value, based on the HC results (mean ± 3 SDs, 21.23 FUs); the values for the patients with AIND (mean ± 3 SDs, 21.17 FUs) and the patients with MS (mean ± 3 SDs, 23.85 FUs) were not different from the HCs, and all were less than 25 FUs. Positive values were found in 19 of 25 patients with NMO (76%) and in 6 of 11 patients with LETM (55%) (Figure 3 and Table 2). The FIPA gave significantly higher results in serum samples that were positive for indirect immunofluorescence (P < .001, Mann-Whitney test) (Figure 3D) and was overall more sensitive and highly specific. Results of the quantitative cell-based assay and FIPA were strongly correlated (P < .001, data not shown).
No correlation was found (P=.72) between the spinal cord magnetic resonance imaging lesion length and the AQP4 antibody values, but a trend was seen toward lower FIPA and cell-based assay values in patients immunosuppressed before serum analysis (P=.07; data not shown). However, antibody levels in patients during relapse were 100% positive, and were higher in patients during relapse than in those sampled during remission (P=.02, cell-based assay; P=.006, FIPA; Mann-Whitney test) (Figure 6A and B).

The clinical classification of the patients in Table 1 is based on their eventual clinical diagnoses and did not incorporate knowledge of their NMO IgG (or AQP4 antibody) status. In 3 patients initially diagnosed as having MS, acute disseminating encephalomyelitis, or inflammatory encephalitis, NMO was diagnosed only several years later, in 2 at the postmortem examination (M. Esiri, DPhil, I. Pomeroy, DPhil, S. Viegas, MD, unpublished data, 2007). All 3 patients were clearly
positive for AQP4 antibodies by cell-based assay (scores of 3) and FIPA (>70 FUs) early in their disease course.

Neuromyelitis optica is an immune-mediated neurologic disease that can be severely disabling. Improvement by immunosuppressive treatment and/or plasmapheresis indicates the need to distinguish it from MS and other diseases associated with inflammatory lesions as early as possible, often before a “full house” of clinical features is evident. Previous reports have shown that antibodies to the water channel AQP4 are specifically associated with this condition. We have established a new assay (FIPA) for potential clinical use and compared it with an AQP4-transfected cell-based assay and with detection of NMO IgG. Our results suggest that the antibodies detected by indirect immunofluorescence are directed principally against AQP4 and are best assayed by an antigen-specific test. In this relatively small cohort, the FIPA and cell-based assay showed superior sensitivity (76%-80%) and 100% specificity.

We were not able to confirm the results of the study by Takahashi et al that the AQP4 antibody titer was related to spinal cord lesion length, but our samples were not all taken at the time of magnetic resonance imaging and some were obtained after immunosuppression, which was associated with reduced values. However, antibody levels were positive in all 10 patients sampled during relapse, and higher positivity overall might be expected if serum samples were obtained during a relapse and before commencement of immunosuppression. Nevertheless, it is not yet clear whether the antibodies are detectable in all patients at presenta-
tion or how often high-risk antibody-negative patients should be retested.

The cell-based assay, as previously reported, demonstrates that disease-associated antibodies bind to AQP4 in a naturally folded state when it is located on the cell surface and, thus, accessible to pathogenic antibodies. Indirect evidence for a humoral pathogenesis in NMO comes from histopathologic studies that demonstrate immunoglobulin deposits and complement activation in acute NMO lesions, as well as a pattern-specific intracellular loss of AQP4. Our finding that AQP4 antibody and NMO IgG belong principally to the IgG1 subclass and can activate complement in vitro provides evidence of the potential pathogenicity of this antibody, confirming a recent report that also demonstrated reduction of surface AQP4 expression by endocytosis or degradation and complement activation. Our detection of IgG1 and complement-activating antibodies on brain tissue by immunofluorescence supports the idea that these mechanisms could operate in vivo. Overall, these results strongly support the use of therapies that target humoral immune mechanisms and also the possibility of anti-complement therapy.

Neuromyelitis optica is the first MS-like disease in which a major antigen has been identified, but it is one of a growing number of central nervous system diseases associated with antibodies to cell membrane proteins, particularly ion channels. The approaches described are already being used to establish assays for antibodies to a range of different ion channels and receptors (P.W., D.B., and A.V., unpublished data, 2005-2008) to diagnose and study the widening spectrum of antibody-mediated neurologic diseases.

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Financial Disclosure: Dr Beeson has filed a patent (PCT/GB2006/050455) for the use of EGFP-tagged acetylcholine receptor, muscle-specific kinase, and AQP4 for autoantibody detection; and Dr Vincent and her department receive royalties and revenue for performing antibody assays for neurologic diseases.

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REFERENCES