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.

Antibodies to different ion channels have been found in several neurologic diseases, and their measurement is clinically important.1,2 A newly defined autoimmune channelopathy is neuromyelitis optica (NMO).3,4 Neuromyelitis optica is an inflammatory disease that involves the spinal cord and optic nerves5,6; it often follows a more aggressive clinical course than multiple sclerosis (MS) but responds better to immunosuppressive therapy.7,8

A need exists for a diagnostic test to distinguish reliably NMO from MS at onset, when the full clinical features may not be apparent but early intervention may prevent disability. In 2004, Lennon et al8 described an NMO IgG antibody using indirect immunofluorescence on adult mouse cerebellum sections, showing a characteristic pattern of binding around microvessels, pia, and Virchow-Robin spaces; it was 58% to 73% sensitive and 91% to 100% specific for NMO.8 This report led to the adoption of NMO IgG positivity as 1 of 3 supportive criteria for the diagnosis of the disease.9 In 2005, the same group identified aquaporin-4 (AQP4) as a target antigen,3 showing binding of some NMO IgG–positive serum samples to the surface of human embryonic kidney (HEK) 293 cells transfected with human AQP4, subsequently confirmed in a study from Japan10 and immunoprecipitation of sulfonilized enhanced green fluorescent protein (EGFP) AQP4 by Western blotting of the precipitates.3 In addition, 57% of NMO cases were positive by immunoprecipitation of in vitro–translated, sulfur 35–labeled methionine AQP4,11 but it is unlikely that the antigen adopts its full native conformation with this technique.

We used EGFP tagging3 to demonstrate a novel, sensitive, quantitative, and highly specific fluorescence immunoprecipitation assay (FIPA) for AQP4 antibodies. In this article, we compare those re-
results 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.\textsuperscript{12}

**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 al\textsuperscript{9} but discounting the criteria of McDonald et al\textsuperscript{13}; 27 with fulfilling the typical NMO immunofluorescence pattern.\textsuperscript{8} For IgM 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 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 37°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 488–conjugated secondary antibody (Invitrogen–Molecular Probes, Paisley, Scotland) 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, Birmingham, England) and then Alexa Fluor 488–conjugated donkey anti–sheep IgG (1:750; Invitrogen Ltd, Paisley, England) was added as a secondary antibody. Slides were mounted as described herein.

**INDIRECT IMMUNOFLUORESCENCE ASSAY**

The NMO IgG was detected by indirect immunofluorescence on 10-µm adult mouse cerebellum cryosections as previously described.\textsuperscript{14} Sections were classified as positive if they exhibited the typical NMO immunofluorescence pattern.\textsuperscript{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 (1:750; 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’-GCATCCCGGGTCATCTAAGACAAATACCTCTCCAG and the forward primer 5’-GTCATCTCGAGATGAGTACGACGCACACCAGCACAGAAG to yield plasmid EGFP-AQP4-M1, or 5’-GTCACTCGAGATGAGTACGAGCTTCAAGG GGTTCTG to yield plasmid EGFP-AQP4-M23.

**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 (DakoCytomation) containing DAPI (1:1000). Mouse brain sections were immunostained for C3b in a similar fashion, except that the HEK.
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). 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>
<thead>
<tr>
<th>Diagnosis</th>
<th>In-house Indirect Immunofluorescence</th>
<th>Cell-Based Assay</th>
<th>FIPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMO (n=25)</td>
<td>14 (58)(^a)</td>
<td>20 (80)</td>
<td>19 (76)</td>
</tr>
<tr>
<td>LETM (n=11)</td>
<td>5 (50)(^b)</td>
<td>6 (55)</td>
<td>6 (55)</td>
</tr>
<tr>
<td>MS (n=38)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AIND (n=26)</td>
<td>1 (4)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HCs (n=14)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Abbreviations:** AIND, autoimmune inflammatory disease; FIPA, fluorescence-based immunoprecipitation assay; HCs, healthy controls; LETM, longitudinally extensive transverse myelitis; MS, multiple sclerosis; NMO, neuromyelitis optica.

\(^a\) The indirect immunofluorescence assay was first established\(^d\) using 3 NMO serum samples (of 8) that were positive for NMO IgG testing by the Mayo Clinic, Rochester, Minnesota.

\(^b\) 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).

To confirm that the antibodies bind to extracellular epitopes on AQPO4, we expressed AQPO4 in HEK 293 cells and used immunofluorescence to detect binding of serum antibodies. Because the EGFP-AQPO4 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 AQPO4 ANTIBOERIES USING THE CELL-BASED ASSAY

All positive serum samples tested, including those with both high and low AQPO4 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 AQPO4 antibody–positive serum samples showed strong complement C3b deposition on the cell membrane (Figure 4A and B), which correlated weakly (\(r^2=0.4, P=.05\)) with IgG1 AQPO4 antibodies (Figure 4C).

### AQPO4 ANTIBOERIES MEASURED BY FIPA

To establish a quantitative and objective assay, we used immunoprecipitation. The EGFP-AQPO4, extracted in detergent from the cells, demonstrated a sedimentation coefficient of 7.4, corresponding to that predicted for an EGFP-AQPO4 tetramer (Figure 5A). Serum samples that were positive for AQPO4 antibodies immunoprecipitated more EGFP-AQPO4 with increasing concentrations of EGFP-AQPO4 (Figure 5B), suggesting relatively low overall affinity. Serum samples that immunoprecipitated EGFP-AQPO4 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-AQPO4 (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-AQPO4 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 3 SDs, 23.85 FUs). More positive results were found with the cell-based assay and FIPA were strongly correlated (\(P<.001\), Mann-Whitney test) (Figure 5D) 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).
TITER OF AQP4 ANTIBODIES AND CLINICAL FEATURES

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 restested.

The cell-based assay, as previously reported, demonstrated 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 intraleSIONAL 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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