Quantification and Functional Characterization of Antibodies to Native Aquaporin 4 in Neuromyelitis Optica

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**Background:** Antibodies targeting membrane proteins play an important role in various autoimmune diseases of the nervous system. So far, assays allowing proper analysis of such autoantibodies are largely missing. A serum autoantibody to aquaporin 4 (AQP4) is associated with neuromyelitis optica (NMO). Although several assays are able to detect this autoantibody, they do not allow determination of the biological activity of anti-AQP4 antibodies.

**Objective:** To develop a bioassay for quantification and characterization of human anti-AQP4 antibodies.

**Design, Setting, and Participants:** We developed a novel bioassay for quantification and characterization of human anti-AQP4 antibodies based on high-level expression of native AQP4 (nAQP4) protein on the surface of human astroglioma cells. The test was validated in 2 independent cohorts of patients with NMO spectrum disease.

**Results:** We detected anti-nAQP4-IgG with a sensitivity of 57.9% and specificity of 100% in patients with NMO spectrum diseases, suggesting that our bioassay is at least as sensitive and specific as the gold-standard NMO-IgG assay. The anti-AQP4 antibodies belonged predominantly to the IgG1 isotype and bound with high affinity to the extracellular domain of nAQP4. Our data suggest that the autoantibody exerts pathological properties because nAQP4-IgG-positive sera induced cell death of nAQP4-expressing cells by antibody-dependent cellular natural killer cell cytotoxic effect and complement activation. Furthermore, nAQP4-IgG titers strongly correlated with in vitro cytotoxic effect.

**Conclusions:** In NMO, this assay may help to unravel the biological function of anti-nAQP4-IgG. Our findings demonstrate the potential of bioassays to characterize biologically relevant antibodies in human autoimmune diseases.

**Arch Neurol. 2010;67(10):1201-1208**

**EUROMYELITIS OPTICA**

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result. Given the potential functional importance of antibodies targeting conformational epitopes of membrane proteins in other CNS diseases and experimental models, assays that precisely reflect expression of the protein in vivo are desperately needed.

Therefore, we established a bioassay that allows quantification of antibodies to human native AQP4 (nAQP4) expressed in a human astrocytoma cell line (nAQP4-IgG). The assay was validated with well-defined sets of sera and was used to investigate the biological activity of anti-nAQP4-IgG in vitro. Complement- and NK cell-mediated cytotoxic effects were observed with all sera containing antibodies to nAQP4. The strong correlation between antibody titer and in vitro cytotoxic effect suggests that the bioassay reliably detects biologically relevant antibodies in NMO and NMO spectrum diseases. Cell-based assays are powerful tools to investigate antibody responses to membrane proteins and may help identify and characterize antibody responses relevant in other autoimmune diseases.

**METHODS**

**PATIENTS AND CONTROLS**

For initial validation of the assay, sera from 14 patients with NMO or opticospinal multiple sclerosis (MS) (13 women, 1 man) participating in clinical studies at the University of California, San Francisco were used. Patients with opticospinal MS were defined as those meeting International Panel Criteria for MS but having clinical attacks restricted to the optic nerves or spinothalamic tracts. The cohort comprised 18 patients fulfilling the diagnostic criteria by Wingerchuk et al, with the exception of NMO-IgG (20 women, 7 men; age range, 18-66 years; median age, 41 years), 12 patients with RION (all women; age range, 20-79 years; median age, 44 years), 16 patients fulfilling the revised McDonald criteria by Wingerchuk et al, with the exception of NMO-IgG (14 patients with RION; age range, 18-71 years; median age, 42 years), and 17 patients with LETM (20 women, 7 men; age range, 24-72 years; median age, 44 years). This cohort was compared with 38 patients with MS (25 women, 13 men; age range, 18-66 years; median age, 41 years) fulfilling the revised McDonald criteria and 64 patients with other neurological diseases (ONDs) (16 women, 48 men; age range, 20-79 years; median age, 41 years) who were recruited in Germany. Patients with ONDs had human immunodeficiency virus infection, various extracerebral or intracerebral neoplasms, viral meningitis, headache, (pseudo)radicular pain, or vertigo.

**CLONING AND EXPRESSION OF AQP4**

The human M1 and M23 variants of AQP4 were expressed by a lentiviral transfection system in the LN18 cell line as described previously. The LN18 cell line is a human glioma cell line established from an astrocytoma biopsy specimen. Total human brain RNA (BD Biosciences, San Jose, California) was used to synthesize full-length complementary DNAs of the M1 and M23 AQP4 variants (with SpeI and SacI restriction sites at 5’ and 3’ ends). The sequence of the common reverse primer used for both M1 and M23 variants was 5’-CCG GGG GTC TTC AGT AGT GGA CGT GTA CTC TGG CTG-3’ for the M1 variant and 5’-ACT AGT ATG GTG GCT TTC AAA GGG GTC TGG-3’ for the M23 variant. The polymerase chain reaction–amplified products were cloned into the plasmid pLenti6/V5 (Invitrogen Corp, Carlsbad, California) by using SpeI and SacI restriction sites. To generate gene-containing virus particles, the pLenti6/V5-AQP4 M1 or M23 constructs and packaging mix were used to transfect a 293 FT cell line by Lipofectamine 2000 (Invitrogen Corp). After transfection of 48 hours, virus-containing supernatant was collected and used to transduce the human LN18 astrocytoma cell line to generate both M1- and M23-expressing cell lines (LN18M1 and LN18M23-AQP4, respectively). The LN18 cell line was also transduced with an empty vector pLenti6/V5 to establish a control cell line (LN18CTR). The 3 stably transduced cell lines were maintained under the same culture conditions and the same selection pressure throughout the experiments.

**INDIRECT IF STAINING**

The LN18M1 and LN18CTR cells were fixed for 10 minutes on ice with cytostix (BD Biosciences). After fixation, cells were blocked for 1 hour with blocking buffer containing 10% normal goat serum (Vector Laboratories, Inc, Burlingame, California). Extensive washing was performed at each step with washing buffer. Cells were incubated with polyclonal rabbit antirat AQP4 IgG antibody (Sigma-Aldrich Co, St Louis, Missouri) in 1X phosphate-buffered saline (PBS). After blocking, cells were incubated with polyclonal rabbit antirat AQP4 IgG–positive serum (diluted 1:1000 in blocking buffer). Cells were incubated for 2 hours on ice, washed 5 times with washing buffer, and incubated for 1 hour with Alexa Fluor-488–labeled goat antirabbit IgG or goat antihuman IgG (Molecular Probes; Invitrogen Corp). The nuclei of the cells were counterstained with 4’,6-diamidino-2-phenylindole (Invitrogen Corp). Images were captured and analyzed with a fluorescence microscope (Fluorescence Microscope System, Carl Zeiss AG, Jena, Germany; or cell^R Imaging Station, Olympus, Essex, England).

**WESTERN BLOT ANALYSIS**

The LN18M1 and LN18CTR cells were lysed with RIPA buffer (Sigma-Aldrich Co). Five micrograms of total protein lysates of LN18M1 and LN18CTR was separated by using 10% Bis-Tris gels (Invitrogen Corp), and proteins were transferred to polyvinylidene fluoride membranes (Invitrogen Corp). The polyvinylidene fluoride membranes were blocked in blocking buffer (1X PBS containing 5% milk and 0.05% Tween 20) for 1 hour at room temperature. The membranes were probed with rabbit polyclonal anti-AQP4-IgG antibody (stock [1 mg/mL]) diluted in blocking buffer (diluted 1:2000). Membranes were incubated overnight at 4°C, washed 5 times with washing buffer (0.05% Tween 20 in 1X PBS), and incubated for 1 hour at room temperature.
FLOW CYTOMETRY ANALYSIS

Flow cytometry was used first to confirm the expression of AQP4 in the LN18\textsuperscript{AQP4} cell lines and second to identify anti-AQP4 autoantibodies in patients’ sera.

The expression of AQP4 in LN18\textsuperscript{AQP4} cells was determined by intracellular staining (for intracellular staining, cells were fixed and permeabilized with Cytofix and Cytoperm \([1\times]\) buffers [BD Biosciences], respectively) with rabbit anti-AQP4 polyclonal IgG antibody and Alexa Fluor-488–labeled goat antirabbit IgG antibody. Surface expression of AQP4 was determined by staining with an NMO-IgG–positive serum in combination with Alexa Fluor-488–labeled goat antihuman IgG antibody (Molecular Probes; Invitrogen Corp.).

We added 20,000 LN18\textsuperscript{AQP4} or LN18\textsuperscript{CTR} cells in 20 µL of RPMI growth medium in duplicate to a U-shaped 96-well plate (CellStar; Greiner Bio-One, Monroe, North Carolina), each well containing 30,000 LN18\textsuperscript{CTR} or LN18\textsuperscript{AQP4} cells in 40 µL of RPMI medium (final IgG concentration was 125 µg/mL) and incubated on ice for 25 minutes on an orbital shaker. Cells were then washed twice with FACS buffer (1% fetal calf serum in 1\% FBS) and RPMI medium, and transferred to a 96-well cell culture plate (F-bottom, CellStar). We added 60,000 CD56\textsuperscript{+} NK cells resuspended in 40 µL of RPMI medium to each well, and RPMI medium was used as the readout in all experiments.

To detect nAQP4-IgG antibodies in clinical samples, 20,000 LN18\textsuperscript{AQP4} and LN18\textsuperscript{CTR} cells in 20 µL of RPMI medium were added to each well containing 20 µL of 1:100 diluted primary antibody or NMO-IgG–positive serum. The plates were incubated on ice for 25 minutes on an orbital shaker. Cells were then washed twice with FACS buffer (1\% FBS containing 1% fetal bovine serum). Alexa Fluor-488–labeled goat antirabbit IgG (Fc) or antihuman IgG (H+L) antibody (diluted 1:100 in FACS buffer) was added to each well and incubated on ice. After 25 minutes, cells were washed twice, resuspended in 175 µL of FACS buffer, and transferred into FACS tubes (BD Biosciences). Cell number and viability were determined by flow cytometry (CytAn ADP) based on forward scatter and sideward scatter parameters.

COMPLEMENT-MEDIATED CYTOTOXICITY ASSAY

On a U-shaped 96-well plate (CellStar), each well was loaded with 50,000 LN18\textsuperscript{CTR} or LN18\textsuperscript{AQP4} cells in 40 µL of RPMI medium. Total IgG from nAQP4-IgG–positive and nAQP4-IgG–negative sera was purified as described earlier, diluted in 40 µL of RPMI growth medium, and added to each well to give a final IgG concentration of 125 µg/mL, in duplicate. Cells were incubated on ice for 25 minutes on an orbital shaker, washed twice with FACS buffer (1% fetal calf serum in 1\% FBS) and once with RPMI medium, and transferred to a 96-well cell culture plate (F-bottom, CellStar). A total of 7.5 µL of nAQP4-IgG–negative serum was added as a complement source in each well, and RPMI medium was adjusted to a final volume of 150 µL (final dilution of control serum was 1:20). After incubation at 37°C in a humidified carbon dioxide incubator for 12 hours, the supernatants were discarded. Remaining cells were detached by adding 70 µL of 0.05% trypsin-EDTA and transferred into FACS tubes (BD Biosciences). Cell number and viability were determined by flow cytometry (CytAn ADP) based on forward scatter and sideward scatter parameters.

RECOMBINANT EXPRESSION OF nAQP4 IN A HUMAN ASTROCYTOMA CELL LINE

To investigate antibody reactivity to nAQP4, we expressed the human M1 AQP4 gene in the human astrocytoma cell line LN18 (LN18\textsuperscript{AQP4}) by a lentiviral expression system (expression and detection strategy displayed in Figure 1A). In parallel, the LN18 cell line was transduced with an empty vector to establish an appropriate control cell line (LN18\textsuperscript{CTR}). Expression of AQP4 in LN18\textsuperscript{AQP4} but not LN18\textsuperscript{CTR} was demonstrated by Western blot (Figure 1B) using a polyclonal rabbit anti-AQP4-IgG (Fc) antibody. A high level of AQP4 expression was confirmed by immunocytochemistry and flow cytometry (Figure 1C and D) on permeabilized LN18\textsuperscript{AQP4} cells.

Second, we verified the cell surface expression of AQP4 on LN18\textsuperscript{AQP4} by incubating AQP4–transfected cells with an NMO-IgG–positive serum (diluted 1:100). Immunocytochemistry with a fluorescence-labeled antihuman IgG–specific secondary antibody revealed appropriate labeling of LN18\textsuperscript{AQP4} but not LN18\textsuperscript{CTR} cells (Figure 2A). Binding of serum NMO-IgG could also be visualized by
flow cytometry in LN18AQP4 but not LN18CTR cells, confirming the suitability of the detection system (Figure 2B, lower panel). Similar results were obtained with the LN18 cell line transfected with the M23 variant of AQP4 (data not shown).

FLOW CYTOMETRY AND WESTERN BLOT:

Polyclonal rabbit and anti-AQP4 antibody/ polyconal goat antirabbit IgG antibody

Figure 1. Bioassay to determine antibodies to human native aquaporin 4 (AQP4). A, Strategy to establish the bioassay for the detection of antibodies binding to native AQP4. The glioma cell line LN18 was stably transduced with either the M1 isoform of the AQP4 gene (to establish the LN18AQP4 cell line) or an empty vector (to establish the LN18CTR control cell line) using a lentiviral expression system (the LN18AQP4-M23 cell line expressing the M23 variant of AQP4 was produced likewise). The different antibodies used in the experiments are displayed. bp indicates base pairs. Expression of AQP4 was confirmed by Western blot of LN18AQP4 and LN18CTR cell lysates (primary antibody: polyclonal rabbit anti-AQP4 antibody; secondary antibody: goat antirabbit IgG) (B), immunocytochemistry (C), and flow cytometry on permeabilized cells (primary antibody: polyclonal rabbit anti-AQP4 antibody; secondary antibody: goat antirabbit IgG) (D).

QUANTIFICATION OF SERUM ANTIBODIES TO nAQP4 BY A CELL-BASED BIOASSAY

Serum antibodies from healthy control donors did not bind to either of the 2 cell lines (Figure 2B, upper panel). Serial dilution of sera from NMO-IgG–positive patients demonstrated that the antibody to nAQP4 can be detected at a dilution of up to 1:100,000 (Figure 2C and D). After logarithmic transformation, dose-response curves were approximately linear (Figure 2D). This indicates that the bioassay is able to detect nAQP4-IgG autoantibodies in serum samples in a quantitative manner over a wide range of concentrations.

COMPARATIVE ANALYSIS OF NMO-IgG AND ANTI-nAQP4-IgG

Next, we wished to validate our assay in comparison with an already established assay for the detection of NMO-IgG. Thus, our assay was applied to 14 serum...
samples from patients with clinically defined NMO or optocarpal MS that had been analyzed at the Department of Laboratory Medicine and Pathology, Mayo Clinic by IP and IF assays, the gold-standard approaches for the detection of NMO-IgG (Table). Eight samples tested positive by IF and IP, 1 sample tested positive by IP but not IF, and 5 samples were negative in both assays. In our assay, all 9 NMO-IgG-positive samples were strongly positive, although titers differed by more than 1 order of magnitude. Interestingly, the difference in the MFI (ΔMFI) of the IP-positive/IF-negative serum was in the midrange. Further, 4 of 5 NMO-IgG–negative sera were below the cutoff of our assay as well, but 1 NMO-IgG–negative serum tested strongly positive. Although these data are derived from a rather small sample set and are thus preliminary, the results suggest that our cell-based assay is comparable to the current gold-standard NMO-IgG assay in terms of sensitivity and specificity.

Similar results were obtained with the LN18 cell line transfected with the M23 variant of AQP4 (LN18AQP4-M23), although serum antibody binding seemed to be slightly lower with this cell line compared with the LN18AQP4 cell line (data not shown). Therefore, all further analyses were performed with the LN18AQP4 cell line.

PREVALENCE OF ANTI-nAQP4-IgG IN NMO SPECTRUM DISEASES

To determine the sensitivity and specificity of our assay, we applied the assay to masked serum samples from patients with NMO, LETM, RION, MS, and OND (Figure 3A). Sera were diluted 1:100 and tested in duplicates using ΔMFI as compared with background staining as the readout.

To define a cutoff for anti-nAQP4-IgG antibody positivity, sera from 64 patients with ONDs were measured as negative controls. The ΔMFI ranged from −8.1 to 5.0, with a median of −0.63. The cutoff was thus set at a ΔMFI of 10, corresponding to twice the 99th percentile of the OND control group.

No sera from patients with MS tested positive for anti-nAQP4-IgG (median ΔMFI, −0.81; range, −8.3 to 5.1). In contrast, anti-nAQP4-IgG antibodies were present in 11 of 18 patients with clinical NMO (61.1%; median ΔMFI, 186; range, −3.5 to 1638.5), 5 of 12 patients with RION (41.7%; median ΔMFI, 1.8; range, −3.3 to 1414.0), and 10 of 27 patients with LETM (37.0%; median ΔMFI, 3.6; range, −6.7 to 1413.8). In 22 of 27 anti-nAQP4-IgG–positive patients, serum concentrations were considered high because they exceeded a ΔMFI of 100 (Figure 3A). Low titers were observed in 4 patients with LETM and 1 patient with NMO. The specificity and positive predictive value of our assay were 100%. The sensitivity was 57.9% with respect to NMO spectrum diseases; the negative predictive value was 66.6%. In line with previous findings, the highest number of patients with anti-nAQP4 antibodies and the highest titers were observed in the NMO group.

ISOTYPE OF ANTI-nAQP4-IgG

To determine the isotype of the anti-nAQP4-IgG, we analyzed all positive serum samples using secondary antibodies specific for human IgG1, IgG2, IgG3, and IgG4. Significant levels of nAQP4-specific antibodies were found only when the secondary antibody was targeted IgG1 (Figure 3B). In line with this finding, a strong correlation between the levels of anti-nAQP4-IgG and anti-nAQP4-IgG1 was observed (Figure 3C). IgM antibodies to nAQP4 were not found in any of the anti-nAQP4-IgG–positive patients at a serum dilution of 1:100.

DETECTION OF ANTI-nAQP4-IgG IN CEREBROSPINAL FLUID

To address the question of intrathecal production of anti-nAQP4-IgG antibodies, we determined the antibody index (AI). The AI is described by the formula AI = Qspec/QIgG, with Q representing the cerebrospinal fluid (CSF)/serum quotient of nAQP4-IgG antibodies (Qspec) or total IgG (QIgG). An AI greater than 1.5 indicates a production of antibodies in the CNS compartment by sequestered plasma cells or plasma blasts (intrathecal synthesis of the specific antibody). Corresponding CSF samples from anti-nAQP4-IgG–positive sera were available from 7 patients with NMO spectrum diseases in whom CSF and serum samples had been collected at the same time. After adjusting for total IgG concentration (QIgG = 1), pairs of serum and CSF samples were subjected to the nAQP4 assay in parallel. In 3 of 7 paired samples, the AI was between 1.0 and 1.5; in the remaining 4 paired samples, the AI was below 1.0 (Figure 3D). Thus, we have no evidence of intrathecal nAQP4-IgG synthesis in this preliminary analysis.
BIOLOGICAL ACTIVITY OF ANTI-nAQP4-IgG

To investigate whether nAQP4-IgG is biologically active, we tested the serum IgG antibodies for their ability to induce a cytotoxic effect in LN18AQP4 glioma cells in the presence of complement or human NK cells (Figure 4). In the presence of NK cells, IgG from anti-nAQP4-IgG–positive sera induced a specific, dose-dependent cytotoxic effect in LN18AQP4 cells but not LN18CTR cells (Figure 4A and B). This effect was evident even at an IgG concentration of 1.25 µg/mL (Figure 4A). The extent of NK cell–mediated cytotoxic effect strongly correlated with the serum concentration of anti-nAQP4-IgG (r = 0.8763; P = .01) (Figure 4C). We also observed a complement-mediated cytotoxic effect in LN18AQP4 cells incubated with IgG purified from anti-nAQP4-IgG–positive sera (Figure 4D). The extent of killing was less pronounced compared with the NK cell–mediated killing but again correlated well with the serum levels of anti-nAQP4-IgG (r = 0.9515; P = .001).

We present data of our novel bioassay for the detection of nAQP4-IgG. The advantage of our assay is 2-fold. First, the
Aquaporin 4 is hypothesized to be the major autoimmune target in NMO spectrum diseases. It is a membrane protein with several transmembrane domains that exists in at least 2 isoforms. It is expressed in astrocytes but also in the distal collecting ducts of the kidney and parietal cells of the stomach. Antibodies to AQP4 are found in a significant proportion of patients with NMO, RION, and LETM but not in those with MS or other autoimmune diseases of the CNS. Interestingly, autoimmunity seems to exclusively target AQP4 in the CNS as neither renal nor parietal cell dysfunction has been described in patients with NMO. This may suggest that the AQP4 protein expressed in the brain differs from the protein expressed outside the CNS. It is well known that tissue-specific posttranslational modifications that alter protein structure and function may occur. In the case of NMO spectrum diseases, expression of AQP4 in astrocytes may render the protein particularly immunogenic. Alternatively, astrocytes might be more vulnerable to anti-AQP4 antibody–mediated cytotoxic effect. Both conditions may account for the CNS-confined autoimmune reaction observed in NMO spectrum diseases.

To reflect the posttranslational modifications that might occur in astrocytes, we established a novel bioassay using a human AQP4-transfected cell line of glial origin. The glial cell line allowed us to express AQP4 protein at high levels on the surface of the cells, possibly with the posttranslational modifications occurring in astrocytes. This bioassay is at least as sensitive as the gold-standard assay, which was used to identify NMO-IgG. Its sensitivity and specificity also seem comparable to or even better than those of other assays that are based on transient transfection of AQP4 in cells of nonglial origin. Moreover, the flow assay allows high-throughput analysis and quantification of antibody titers to AQP4, which simplifies testing for this autoantibody.

The new assay allowed characterization of the isotype and biological activity of antibodies that target AQP4. We confirmed the high prevalence of anti-nAQP4-IgG autoantibodies in a cohort of patients with NMO spectrum disease who had been selected solely on the basis of clinical criteria. The antibody response to nAQP4 was almost exclusively IgG1. We found little evidence for a quantitative production of AQP4-specific antibodies in the CSF compartment, although locally produced antibodies may rapidly bind AQP4 in the CNS and thus escape detection by the assay. The AQP4-positive sera very efficiently induced complement- and NK cell–dependent cytotoxic effects of AQP4-expressing glioma cells. Interestingly, nAQP4-IgG concentrations correlated strongly with the extent of cytotoxic effect, suggesting that the antibodies detected with our assay represent the biologically active fraction of antibodies targeting AQP4 in vivo. It is possible that the serum anti-AQP4-IgG concentration might be useful to monitor disease activity in NMO spectrum diseases. It is yet unclear why one-third of patients with NMO spectrum disease are AQP4 antibody negative in our assay. It is still possible that AQP4 may undergo posttranslational modifications and clustering, which are not achieved even in a transfected tumor cell line derived from astrocytes. Alternatively, these AQP4 antibody–negative patients with NMO may have another yet unknown autoantigen targeted in the CNS.

Besides the implications for the understanding of NMO spectrum diseases, our findings underscore the need for improved assay systems for the detection of autoantibodies that target membrane proteins in human autoimmune diseases. The importance of antibodies targeting native vs denatured proteins was demonstrated in experimental autoimmune encephalomyelitis models induced by human myelin oligodendrocyte glycoprotein. While antibodies reactive to both denatured and native myelin oligodendrocyte glycoprotein are found in myelin oligodendrocyte glycoprotein–immunized animals, only antibodies targeting native myelin oligodendrocyte glycoprotein can mediate demyelination in vivo. Therefore, expression systems displaying the protein in the proper conformation are desperately needed to identify antibodies of relevance for human autoimmune diseases. Such bioassays not only allow more accurate antibody measurement but also enable determination of their biological activity with respect to various mechanisms of antibody-mediated cytotoxic effect. The implementation of such bioassays for the search for autoantibodies in human autoimmune diseases might allow us to discover and characterize antibodies that have escaped identification by screening with conventional assays.

References


Accepted for Publication: February 25, 2010.

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Financial Disclosure: None reported.

Funding/Support: This work was supported by grant He2386/7-1 from Deutsche Forschungsgemeinschaft and the Kompetenznetz Multiple Sklerose (Control-MS). Dr Bennett was supported by grant RG4320 from the National Multiple Sclerosis Society.


