Diagnosis of Neuromyelitis Spectrum Disorders

Comparative Sensitivities and Specificities of Immunohistochemical and Immunoprecipitation Assays

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Objective: To compare the sensitivity and specificity of immunofluorescence (IF) and immunoprecipitation (IP) assays using green fluorescent protein–tagged aquaporin-4 (AQP4) in 6335 patients for whom serological evaluation was requested on a service basis.

Design: Case-control study.

Setting: Mayo Clinic Neuroimmunology Laboratory (Rochester, Minnesota) and Departments of Neurology (Rochester, Minnesota; Scottsdale, Arizona; and Jacksonville, Florida).

Patients: Group 1, 835 Mayo Clinic patients, 100 with a neuromyelitis optica (NMO) spectrum disorder diagnosis and 735 without NMO spectrum disorder; group 2, 5500 non–Mayo Clinic patients.

Main Outcome Measure: Sensitivity and specificity of each assay for NMO or NMO spectrum disorder, individually and combined.

Results: In group 1, the sensitivity rates for NMO were IF, 58%; IP, 33%; and combined assays, 63%. The sensitivity rates for relapsing longitudinally extensive transverse myelitis were IF, 29%; IP, 6%; and combined assays, 29%. The specificity rates for NMO and relapsing longitudinally extensive transverse myelitis were IF, 99.6%; IP, 99.3%; and combined assays, 99.2%. In group 2, NMO-IgG was detected by IF in 498 of 5500 patients (9.1%) and by IP in 331 patients (6.0%); 76 of the 331 patients seropositive by IP (23%) were negative by IF. Clinical information was available for 124 patients (including 16 of those seropositive by IP only); 123 had a definite NMO spectrum disorder and 1 was at risk for NMO (monophasic optic neuritis).

Conclusions: In this large, clinical practice-based study, NMO-IgG detected by IF or IP was highly specific for NMO spectrum disorders. The IP assay was significantly less sensitive than IF. Combined testing improved sensitivity by 5%.

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ficity of NMO-IgG for diagnosis of NMO spectrum disorders.15-18 Recent studies suggest that IP and cell-binding assays for AQP4-specific antibodies are more sensitive to NMO spectrum disorders than tissue-based IF.16,19 From their experience testing the sera of 36 patients with NMO spectrum disorders, Waters et al19 reported in the Archives that a GFP-linked AQP4 IP assay and a cell-based AQP4 IF assay were more sensitive than a tissue-based IF (respective sensitivities, 76%, 80%, and 58%). The authors reported specificity rates of 96% for IF and 100% for IP. Our experience does not concur with theirs. We present here the results of a blinded side-by-side comparison of IF and IP for detecting NMO-IgG in the sera of 6335 patients tested on a clinical service basis.

PATIENTS

Group 1 consisted of 835 adult patients for whom service NMO-IgG testing was requested from January 2004 through December 2007; 559 were women (67%). All were evaluated clinically at 1 of Mayo Clinic’s 3 sites (Rochester, Minnesota; Scottsdale, Arizona; or Jacksonville, Florida). Clinical information was obtained by medical record review. The treating neurologist and the clinician reviewing the medical records (A.M. and S.J.P.) were both blinded to each patient’s serological status at the initial clinical evaluation. Two patients whose neurological referral to Mayo Clinic followed NMO-IgG detection were excluded from the study. Patients were classified on the basis of initial clinical findings (excluding autoimmune serology) as having an NMO spectrum disorder (n=100) or another neurological diagnosis (n=735). Diagnoses of NMO spectrum disorder included NMO (n=40; all met 2006 revised clinical criteria, with exclusion of NMO-IgG seropositivity as a supportive criterion20), LETM (n=43; 17 had 2 or more episodes), and recurrent optic neuritis (n=17). The 735 patients with other neurological diagnoses had classic multiple sclerosis (n=249), transverse myelitis (n=64; <3 vertebral segments); monophasic optic neuritis (n=40), acute disseminating encephalomyelitis (n=6), and miscellaneous neurological disorders (n=376; 7 had other unspecified monophasic central nervous system demyelinating disorders).

Group 2 consisted of 5500 adult patients not seen at Mayo Clinic for whom NMO-IgG testing was requested through the Mayo Neuroimmunology Laboratory, October 1, 2007, through March 31, 2008. Clinical information was available for 124 patients.

SEROLOGICAL TESTING

Indirect Immunofluorescence

The substrate consisted of nonpathological mouse brain and kidney tissues. Serum was preabsorbed at 1:120 dilution with liver powder to reduce interference from non-organ-specific autoantibodies. Tissue-bound IgG was detected using fluorescein-conjugated goat antihuman IgG. Seropositivity (Figure) was scored when IgG bound selectively to AQP4-rich tissues (both glia limitans and a subset of distal collecting tubules in the renal medulla).13

AQP4 Immunoprecipitation

Duplicate aliquots of recombinant human GFP-AQP4 (150 000 GFP counts, solubilized from human embryonic kidney [HEK]-293 cells transfected with plasmid-encoding enhanced GFP [pEGFP] C2-AQP4) were held with 30 µL of serum for 16 hours at 4°C.3 Control sera included 4 pools of normal human serum and a single AQP4-IgG–positive serum. Two hours after adding protein-G Sepharose 4B (30 µL) (Zymed Laboratories, South San Francisco, California), beads were washed with lysis buffer and bound GFP was measured spectrophotometrically. After subtracting the maximum value yielded by a panel of normal control sera, we calculated GFP-AQP4 bound (in nmol/L serum) by reference to a GFP standard (Clontech, Mountain View, California). Sensitivities and specificities for each possible cutoff for the upper limit of the reference range were plotted on a receiver operating characteristic curve (not shown). The receiver operating characteristic analysis indicated a positive cutoff value of 2.1 nmol/L. At this cutoff value, specificity was compromised because 8 of 14 from group 1 with values of 2.1 to 10.0 nmol/L (57%) lacked an NMO spectrum disorder, and only a 7% improvement in sensitivity was observed. Therefore, a conservative positive cutoff value higher than 10.0 nmol/L was chosen. Sera yielding values higher than 10 nmol/L were clarified by centrifugation (20,000g) and reassayed with GFP-AQP4 and with GFP alone (150 000 counts). The final results represent average GFP-AQP4 values for each patient after subtracting the individuals’ values for precipitation of GFP alone.21
STATISTICAL ANALYSES

The sensitivity and specificity of autoantibody detection by each assay, individually and in combination, was determined by reference to the clinical diagnoses assigned to group 1 patients. The McNemar test was used to compare the significance of differences in sensitivity and specificity between (1) IF and IP and (2) IF and combined assays. \( P < .05 \) was considered significant. The 95% confidence intervals (measures of precision of calculated sensitivities and specificities) were also determined. Analyses were performed using JMP statistical software (version 7.0; SAS Institute Inc., Cary, North Carolina).

This study was approved by the Mayo Clinic institutional review board (IRB 07-007453).

RESULTS

GROUP 1

In the period of comparative assay evaluation, seroprevalence rates for NMO-IgG for all 835 Mayo Clinic patients were 4.2% by IF, 2.4% by IP (median, 45.2 nmol/L; range, 13.5-563.6 nmol/L), and 4.9% by 1 or both assays. Seroprevalence rates for NMO-IgG for 466 Mayo Clinic patients with a diagnosis of an inflammatory demyelinating central nervous system disorder (optic neuritis, transverse myelitis, NMO, multiple sclerosis, acute disseminated encephalomyelitis, other unspecified monophasic) were 7.5% by IF, 4.3% by IP, and 8.8% by 1 or both assays. The NMO-IgG seropositive patients were predominantly female (IF, 31 of 35 patients [89%]; IP, 16 of 20 patients [75%]).

The sensitivities and specificities of the individual and combined assays for diagnosis of NMO, recurrent LETM, and recurrent optic neuritis (and 95% confidence intervals) are documented in Table 1 and Table 2. Neither assay differed with respect to specificity (\( P = .32 \)) for these 3 clinical diagnoses. However, the IF assay was more sensitive than the IP assay for diagnosis of NMO (58% vs 33%; \( P = .008 \)) and relapsing LETM (29% vs 6%; \( P = .04 \)). There was a trend toward superior sensitivity for NMO when the 2 assays were used in combination by comparison with the IF assay alone (63% vs 58%), but this trend did not reach statistical significance.

GROUP 2

Neuromyelitis optica IgG was detected in 574 of 5500 non-Mayo Clinic patients (10.4%) tested during a 6-month period of side-by-side evaluation (Table 3), by IF in 498 patients (9.1%), and by IP in 331 patients (6.2%; median, 47.2 nmol/L; range, 10.1-3661 nmol/L). Of the 331 IP-seropositive patients, 76 (23%) were negative by IF (median, 32.5 nmol/L; range, 10.2-867 nmol/L). Clinical information was available for 124 of 574 seropositive patients (IF or IP); 16 were positive by IP only. Of these patients, 123 had a definite NMO spectrum dis-
order (NMO, 55; LETM, 61; recurrent optic neuritis, 7) and 1 had a monophasic optic neuritis (at risk for NMO).

SEROPOSITIVE PATIENTS NOT FULFILLING CLINICAL DIAGNOSTIC CRITERIA FOR AN NMO SPECTRUM DISORDER

Only 6 of 165 total seropositive patients for whom clinical information was available lacked the signs and symptoms of an NMO spectrum disorder (5 of 41 group 1 patients and 1 of 124 group 2 patients). All 6 were IP positive, and 2 were additionally IF positive (Table 4). Clinical findings in both IF-positive patients and in 1 of the 4 IP-positive patients were nevertheless consistent with an NMO spectrum disorder but, in isolation, were not diagnostic. They had intractable vomiting or monophasic optic neuritis. Clinical diagnoses for the remaining 3 IP-positive patients were idiopathic myelopathy, extremity paresthesia, and progressive multiple sclerosis.

This study, performed in a large clinical practice setting, confirms the high specificity of NMO-IgG as a marker of NMO spectrum disorders, whether detected by IF or by GFP-linked AQ44 IP assay. The lower sensitivity of the IF assay for clinically defined NMO in this study (58% compared with 73%2) likely reflects the study design because patients and sera were acquired consecutively on a clinical basis rather than selected from a pool of known cases and controls. Female sex predominated among NMO-IgG seropositive patients with an NMO spectrum disorder (9:1 vs 2:1 for group 1). Only 1 of 247 patients with multiple sclerosis was NMO-IgG seropositive. Seropositivity by IF had a sensitivity of 58% for NMO among 40 unselected patients with NMO whose sera were consecutively acquired through neurology clinics across 3 Mayo Clinic sites. Of these patients, IF had superior sensitivity to NMO and relapsing LETM compared with IP.

The clinically diverse patients in group 1 were selected for serological evaluation by their treating physician. Their combined 8.8% prevalence of NMO-IgG seropositivity (by IF or IP) indicates that this is not a rare autoantibody. Population-based studies will be required to accurately determine the seroprevalence of NMO-IgG. Group 1 (consisting of 835 Mayo Clinic patients) was too small to demonstrate a statistically significant enhancement of sensitivity with IP as an adjunct to IF testing. However, group 2 (consisting of 5500 non–Mayo Clinic patients), which was more representative of routine clinical practice, demonstrated that IP adds to the sensitivity of optimized IF evaluation for NMO-IgG. In that group, IP yielded an additional 76 seropositive cases over 6 months (13% of 574 total seropositive patients). In this laboratory’s contemporary serological practice (6500 patients tested for NMO-IgG on a service basis in the first half of 2008), IP is a valuable adjunct to the IF assay. Among seropositive patients, most were seropositive by both IF and IP, but others were seropositive by one or another assay alone. This heterogeneous serological profile is consistent with interindividual differences in NMO-IgG–binding avidity to solid-phase AQ44 (immunohistochemical) and liquid-phase AQ44 (IP).

Serositivity in both assays was highly specific for NMO spectrum disorders. To maximize the specificity of the IP assay, we chose a conservative cutoff value of 10 nmol/L. This value identified 6 seropositive patients from groups 1 and 2 whose clinical presentation did not meet the criteria for an NMO spectrum disorder. Two patients who were positive by both IF and IP had presentations that were clinically and biologically consistent with an NMO spectrum disorder, namely intractable nausea and vomiting.23,24 Misu and colleagues23 described medullary MRI abnormalities in 6 of 8 patients with NMO who experienced intractable nausea and hiccoughs. A pathological substrate for this entity has been described: inflammatory foci associated with AQ44 loss from the area postrema.25 Of the 4 patients who were seropositive only by IP, 1 had monophasic optic neuritis, which may have been the first manifestation of an NMO spectrum disorder. Despite extensive evaluations, no clinical, radiological, or electrophysiological evidence of an NMO spectrum disorder was found in the remaining 3 patients who were seropositive only by IP (values were >240 nmol/L in 2 patients and 22.3 nmol/L in 1). The significance of AQ44-binding antibodies in those patients is uncertain. Their clinical diagnoses were primary progressive multiple sclerosis, myelopathy of unknown cause, and paresthesias of the extremities of unknown cause.

We did not compare the utility of our optimized tissue-based IF assay or AQ44 IP assay with IF on a substrate of AQ44-transfected cells. Assays based on this methodology2 may have superior sensitivity; both Waters et al19 and Takahashi et al16 reported a sensitivity of 80% of 35 patients with NMO tested. The assay has not been evaluated in the setting of high-throughput demands of a large clinical laboratory service.

At this stage in the evolution of assays for detecting NMO-IgG in a clinical setting, optimized tissue-based IF assays remain the criterion standard for serological evaluation of a suspected NMO spectrum disorder. In our current practice, the GFP-linked AQ44 IP assay provides an adjunct to immunohistochemical detection of NMO-IgG for moderate enhancement of antibody detection sensitivity.

Table 4. Diagnoses of 6 Seropositive Patients Not Fulfilling Diagnostic Criteria for an NMO Spectrum Disorder (Groups 1 and 2)

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>IF Value, nmol/L</th>
<th>IP Value, nmol/L</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intractable nausea, vomiting</td>
<td>+</td>
<td>30.4</td>
<td>1</td>
</tr>
<tr>
<td>Intractable nausea, vomiting</td>
<td>+</td>
<td>18.8</td>
<td>1</td>
</tr>
<tr>
<td>Myelopathy, cause unknown</td>
<td>−</td>
<td>241</td>
<td>1</td>
</tr>
<tr>
<td>Extremities paresthesia, cause unknown</td>
<td>−</td>
<td>240</td>
<td>1</td>
</tr>
<tr>
<td>Progressive MS</td>
<td>−</td>
<td>22.3</td>
<td>1</td>
</tr>
<tr>
<td>Monophasic ON</td>
<td>−</td>
<td>54.9</td>
<td>2</td>
</tr>
</tbody>
</table>

Abbreviations: IF, immunofluorescence; IP, immunoprecipitation; MS, multiple sclerosis; NMO, neuromyelitis optica; ON, optic neuritis.

Reference value, ≤10 nmol/L.
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**REFERENCES**