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AQP4-IgG Immunoprecipitation Assay Optimization

To the Editor:
The sensitivity and specificity of tissue-based immunofluorescence assays (IFA) is established for detecting neuromyelitis optica (NMO) IgG as an aid to classifying inflammatory demyelinating central nervous system disorders belonging to the NMO spectrum (1): NMO, partial and inaugural forms of NMO [longitudinally extensive transverse myelitis (>3 vertebral segments radiologically) and recurrent optic neuritis] and pediatric inflammatory autoimmune encephalopathies. Immunoprecipitation assays (IPA) employing recombinant human aquaporin-4 (AQP4), the antigen of NMO, have been described (2, 3). In optimizing the green fluorescent protein (GFP)-tagged AQP4 IPA (2) for high-throughput testing, we encountered cases in which patient IgG bound to GFP and not to AQP4. Here we report our efforts, in a clinical setting, to determine the rate of false positivity and take steps to eliminate it.

From October 1, 2007 to March 31, 2008, the Mayo Clinic Neuroimmunology Laboratory evaluated, by use of GFP-AQP4 IPA, sera from 117 healthy adult control individuals (group 1) and from 5500 patients for whom service NMO-IgG IFA testing was requested. Of these, 557 were IFA-positive (group 2) and 4943 were IFA negative (group 3). We also tested neurologically asymptomatic patients, 58 with hypergammaglobulinemia (group 4) and 27 with, systemic lupus erythematosus or Sjögren syndrome (group 5).

In the course of clinical serological evaluation for NMO-IgG (indirect immunofluorescence assay performed on a service basis in the Mayo Clinic Department of Laboratory Medicine and Pathology), patients were identified. Medical records were reviewed retrospectively. The Institutional Review Board (IRB) of the Mayo Clinic College of Medicine, Rochester, MN, approved the study (IRB 07-007453).

We added 30 µL of serum to duplicate 85-µL aliquots of recombinant human GFP-AQP4 [150 000 GFP counts, solubilized in lysis buffer (700 mmol/L NaCl, 10 mmol/L Tris-HCl pH 8.0, 1 mmol/L EDTA, 0.5% Triton X-100)] from human embryonic kidney—293 cells transfected with pEGFP-AQP4 (plasmid-DNA—encoding enhanced GFP-AQP4) (2) and held 16 h at 4°C. Control specimens contained 4 different pools of normal human serum samples or 1 serum sample.
from an AQP4-IgG-positive patient. Two hours after adding protein-G sepharose 4B (30 μL; Zymed Laboratories), we washed the beads 6 times with lysis buffer and measured GFP spectrophotometrically (excitation, 485 nm; emission, 535 nm). After subtracting the maximum value yielded by normal control sera, we calculated GFP-AQP4 bound (nmol/L serum) by reference to a GFP standard (Clontech). Sera yielding values >10 nmol/L serum were clarified by centrifugation and reassayed with GFP-AQP4 and with GFP alone (150 000 counts). The final results represent each patient’s mean GFP-AQP4 value after subtracting the individual’s value for precipitation of GFP alone.

Analysis of serum from groups 1, 4 and 5 yielded a final GFP-AQP4 concentration ≤10 nmol/L serum. In group 2, of 557 sera positive by IFA, none precipitated GFP alone, and 331 yielded initial and finally corrected AQP4-IgG concentrations >10 nmol/L (59%; median 46.6, range 10.1–3661 nmol/L). Clinical information was available for 54 of these 331 patients (16%). All had an NMO-spectrum disorder: 28 patients had NMO, 24 had longitudinally extensive transverse myelitis (recurrent in 12 patients), and 2 had recurrent optic neuritis. In group 3, of 4943 sera that were NMO-IgG negative by IFA, 80 yielded initial AQP4-IgG concentrations >10 nmol/L (1.6%; Fig. 1); 76 remained positive after GFP values were subtracted (median 32.6, range 10.2–867 nmol/L). Diagnoses for the 4 patients who were finally seronegative after GFP values were subtracted (AQP4-IgG range 29–65 nmol/L) were neuroretinitis, primary progressive multiple sclerosis, neurosarcoidosis, or paraneoplastic optic neuropathy. Clinical information was available for 16 of the 76 patients who remained seropositive. Fifteen (93.8%) had an NMO-spectrum disorder [NMO, 3; longitudinally extensive transverse myelitis, 10 (recurrent in 5 patients); recurrent optic neuritis, 2] and 1 had monophasic optic neuritis.

The possibility of false-positive results for AQP4-IgG due to immunoprecipitation of GFP must be considered when sera are evaluated by using recombinant autoantigens fused with this tag. This consideration is of particular importance in patients who are seronegative for NMO-IgG by standardized IFA (group 3 in our study). Among 80 such patients, we obtained a false-positive rate of 5%. After we eliminated those patients from consideration, all but 1 of the remaining 16 patients who had serum samples positive by IPA alone and for whom clinical information was available were confirmed to have a clinical disorder recognized to be in the NMO spectrum. The exceptional seropositive patient had a diagnosis of monophasic optic neuritis. One can reasonably speculate that seropositivity in this case predicts future relapse. False-positive results analogous to those we report for the GFP-AQP4 assay have been reported in immunoprecipitation assays for the muscle nicotinic acetylcholine receptor antibody (125I-α-bungarotoxin tag) (4) and muscle-specific kinase (MuSK) antibody (placental alkaline phosphatase tag) (5). The results of this large study suggest that our optimized high-throughput IPA using GFP-AQP4 is not as sensitive as the originally described tissue-based IFA for detecting NMO-IgG. However, as an adjunct to IFA, the IPA described here enhances the diagnostic yield of NMO-spectrum disorders. A more formal comparison of sensitivity and specificity of IFA, IPA, and combined assays in consecutively-acquired patients with clinical information available in each case is underway. Assurance of assay specificity requires elimination of false-positive results to preclude misdiagnosis and inappropriate long-term commitment of patients to immunotherapies with attendant expense and risks.
Letters to the Editor

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Comparison of Bromcresol Purple and Capillary Protein Electrophoresis for Quantification of Serum Albumin in Multiple Myeloma

To the Editor:

According to the International Staging System (1), serum albumin concentration is a key factor in determining patient prognosis in multiple myeloma. A recent study published in Clinical Chemistry described significant discordance between bromcresol green (BCG)1 albumin dye and agarose gel serum protein electrophoresis (SPEP) for albumin concentration determination in patients with high concentrations of monoclonal (M)-protein (2). Also available, however, are commonly used alternative methods for albumin determination, such as bromcresol purple (BCP) albumin dye and capillary zone SPEP (3). To examine whether the previously observed comparative discordance of measured albumin concentration is limited only to the BCG dye and agarose gel SPEP methods of albumin determination (2), we retrospectively compared measured albumin concentrations as determined by BCP albumin dye and capillary zone SPEP in a large cohort of multiple myeloma patients.

The ratio of measured BCP albumin to SPEP albumin concentrations in patients with a previously identified monoclonal protein was retrospectively calculated for 579 specimens submitted to the University of Arkansas Immunology laboratory for routine serum protein electrophoresis by capillary zone SPEP. All specimens had concurrent albumin determination by a BCP albumin dye method. These consisted of 355 IgG, 122 IgA, 29 IgM, 13 IgD, and 60 free-light-chain myeloma patient specimens. The monoclonal protein light chains in these specimens were approximately evenly divided between κ and λ. Serum albumin was measured by the Beckman-Coulter Synchron BCP assay, and total serum protein was determined by using the Beckman-Coulter Synchron biruuet method on a Beckman-Coulter LX20 Pro modular system. A Sebia Capillarys 2 was used to determine the relative serum sample albumin and M-protein fractions. The relative albumin and M-spike fractions were multiplied by total protein to obtain final SPEP albumin and M-protein concentrations.

Substantial discrepancies in albumin determination in the presence of M-protein between capillary zone SPEP and the BCP albumin determination methods were observed. Although albumin

1 Nonstandard abbreviations: BCG, bromcresol green; SPEP, serum protein electrophoresis; BCP, bromcresol purple; M, monoclonal.