Detection of Acetylcholine Receptor Blocking Antibodies by Flow Cytometry

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Key Words: Myasthenia gravis; AChR antibodies; Flow cytometry

ABSTRACT

Objectives: Detection of acetylcholine receptor (AChR) blocking antibodies through the use of a radiolabel has become standard procedure in most laboratories. Known drawbacks associated with radioassay, including cost of radioisotopes, hazards to laboratory professionals, and manufacture and disposal of radioactive materials, have prompted investigation into replacement assays. We describe here a high-throughput immunofluorescent flow cytometric assay designed for the detection of AChR blocking antibodies.

Methods: In total, 323 serum samples were tested on both the AChR blocking radioassay and the new immunofluorescent flow cytometric assay.

Results: Analysis of the results revealed a 96.9% concordance between the two assay methods.

Conclusions: Our results indicate that a new immunofluorescent flow cytometric AChR blocking antibody assay is not only feasible but clinically comparable in both sensitivity (91%) and specificity (99%) compared with radioassay.

Myasthenia gravis (MG) is a neuromuscular autoimmune disease primarily associated with the presence of antibodies directed toward receptors embedded in the motor endplate at the neuromuscular junction (NMJ). These antibodies cause blockage, dysfunction, and degradation of acetylcholine receptor (AChR), leading to muscle weakness and fatigability. Three main types of AChR antibodies have been identified and are referred to as binding, modulating, and blocking.

These three antibodies are classified based on their different effector mechanisms. AChR binding antibody causes binding and activation of complement at the NMJ. Modulating AChR antibodies cause accelerated degradation of AChR molecules that have been cross-linked by the antibody, a process also referred to as antigenic modulation. Last, the AChR blocking antibody causes functional blocking of the acetylcholine (ACh) binding site.

Blocking antibodies render AChR inoperable due to the functional blockage caused by antibody binding at the normal ACh ligand binding site. This mechanism of AChR disruption is less commonly demonstrable in vitro than binding and modulating of receptors; however, in rodent studies, the administration of blocking antibodies causes an acute and severe form of muscle weakness in the absence of either inflammation or necrosis of the NMJ.

In 2009, Keefe et al described a method of detecting AChR modulating antibodies using flow cytometric techniques. They used fluorescently labeled molecules to aid in the detection of modulated AChR and to confirm the presence of AChR modulating antibodies. A similar protocol...
was created and tested. This protocol demonstrated that similar techniques allow the conversion of the AChR modulating antibody assay to flow cytometry in the clinical laboratory.4

Currently, AChR blocking antibody serologic testing is conducted primarily with the use of a 125I radiolabeled α-bungarotoxin and radioimmunoassay (RIA) method.5 While this assay type has proven to be sensitive in measuring AChR antibodies, using sources of ionizing radiation in the laboratory is expensive and hazardous to both laboratory personnel and the environment. Here, we present a flow cytometric–based method for detecting AChR blocking antibodies through the use of a fluorescently labeled α-bungarotoxin.

Materials and Methods

Study Cohort

Serum samples from 323 individuals were collected and tested. These samples were selected based on three categories, including self-proclaimed healthy donors, screened RIA AChR blocking antibody negatives, and screened RIA AChR blocking antibody positives. The healthy donor population consisted of 120 self-proclaimed, healthy individuals, and serum samples were collected in accordance with University of Utah Institutional Review Board Protocol 7740.

The 203 disease population samples were submitted to ARUP Laboratories for AChR blocking antibody testing and were deidentified and tested in accordance with University of Utah Institutional Review Board Protocol 7275. We did not have access to the clinical history in these deidentified patient samples.

Flow Cytometric AChR Blocking Antibody Detection Assay

Following a novel, laboratory-developed assay protocol. The assay uses the human rhabdomyosarcoma-derived TE671 (RD) cell line, an adherent cell type that expresses fetal acetylcholine receptors.6 These cells were cultured in 175-cm² flasks at 37°C, 5% CO₂, in a 95% humidity incubator to confluence. Culture medium was produced by the ARUP Laboratories Reagent Lab and consisted of Eagle’s minimal essential medium supplemented with 10% heat-inactivated Fetal Clone III, minimal essential medium (MEM) essential and nonessential amino acids, and MEM vitamins.

Cells were harvested from flasks using 1× phosphate-buffered saline (PBS) containing 9 mM EDTA and dispensed into 96-well tissue culture plates at a concentration of 1 × 10⁵ cells per well and were again incubated for 12 to 24 hours at 37°C, in 5% CO₂, and at 95% humidity to allow for cell adherence. Following the overnight incubation, 10 μL controls, patient serum samples, or assay buffer composed of 0.5% bovine serum albumin in Hank’s balanced salt solution without Ca²⁺ or Mg²⁺ were added to the RD cells in designated positions on the plate and then gently agitated before incubating for 2 hours in a standard cell culture incubator.

The plate was then washed twice by slowly pipetting 200 μL assay buffer to each well and then inverting and gently blotting. After washing unbound material from the plate, 100 μL assay buffer and 10 μL of a 1:201 mixture of Alexa-647 (Life Technologies, Carlsbad, CA) labeled α-bungarotoxin and assay buffer were added. The plate was incubated for 60 minutes at room temperature and protected from light. Cells in the well that were intended to serve as background receptor staining were not exposed to serum or α-bungarotoxin.

The unbound material was again washed from the plate following the same wash procedure as stated above. The RD cells were then dissociated from the 96-well plate by adding 100 μL 9 mM EDTA-PBS and incubating at room temperature in the dark for 30 minutes. Using a multichannel pipette, 50 μL assay buffer and 50 μL 1% paraformaldehyde in PBS were added to each well. The contents of each well were vigorously pipetted to dissociate all RD cells. The fixed cell suspension from each well was then transferred to a prelabeled flow cytometry tube. These tubes were then analyzed on a BD FACSCanto II cytometer (BD Biosciences, San Jose, CA) using Diva software.

Data Analysis

A background stain was performed with each flow cytometric run to quantify and subtract inherent cellular fluorescent signal from all control and patient sample geometric means. Maximum staining was obtained by adding only fluorescent label to two wells on each 96-well plate. Both the background and maximum stain geometric means were used to calculate specimen results.

Cell populations were gated to exclude cell fragments and aggregates in the P1 gate. The P2 gate discriminator lines were set on the histogram so that between 1% and 2% of the unlabeled cell population was to the right of the discriminator line [Figure 1A]. The P3 geometric mean fluorescence of this population represents the background signal caused by inherent cellular fluorescence. The geometric mean result of the P2 gate is excluded from the calculation as it is entirely background signal. A right shift of the peak into P3 indicates the presence of the fluorescent marker [Figure 1B].
Percent blocking results were calculated using the population mean fluorescent intensity (equation (1)). The average geometric mean value from the background stain in P3 is subtracted from all other P3 geometric mean readings. A high percent coefficient of variation (CV%) across the two background wells would indicate quality control (QC) issues. The percent blocking result is then calculated as 1 minus the ratio of the patient sample and maximum stain geometric means after background subtraction, multiplied by 100 to obtain a percentage. The fluorescence detected is inversely proportional to the result—meaning, higher fluorescent signal indicates more receptors with no blocking antibody bound.

**Equation 1**

\[
\left(1 - \frac{(P3_{\text{Sample}} - P3_{\text{Background}})}{(P3_{\text{Maximum}} - P3_{\text{Background}})}\right) \times 100\%
\]

**Results**

In this study, we assessed the performance characteristics of the new flow cytometry–based AChR blocking antibody assay relative to those of the currently employed AChR blocking antibody RIA.

Nonparametric statistical analysis was used to evaluate the flow cytometric AChR blocking antibody results of the 120 healthy donors. These results were used to establish a reference interval following Clinical and Laboratory Standards Institute EP28-A3c guidelines. No outliers were observed in this data set.

All 120 healthy donor serum sample results were negative on both RIA and flow assays. The 203-patient serum sample group comprised 102 RIA-negative samples and 101 RIA-positive samples. Of the 102 RIA-negative samples, one sample tested positive by the flow method. The patient with the discordant flow–positive result also tested positive for binding and modulating antibodies. Of the 101 RIA-positive samples, nine tested negative on the flow assay. Two of these nine negative-flow AChR blocking
serum samples did not possess detectible levels of binding or modulating antibodies, whereas three tested positive for binding antibodies only, and the remaining four samples tested positive for both binding and modulating antibodies and were likely false-negative samples in the flow assay.

Threshold values used for qualitative evaluation were calculated by Wilcoxon signed rank, Kruskal-Wallis, and receiver operating characteristic curve statistical analyses. Qualitative results exhibited 96.9% agreement between the currently employed radioassay and the new flow cytometric assay, as shown in Table 1. The remaining samples with discordant results were evaluated further by analyzing AChR binding and modulating results to determine overall agreement.

The observed sensitivity of the new flow blocking assay in relation to the current radioassay is 91%; however, when factoring in the binding and modulating results, this assay demonstrates a higher sensitivity. Specificity of the flow assay in relation to the current radioassay is 99%.

Discussion

MG is a well-characterized neuromuscular, autoimmune disorder. Clinical symptoms of MG are attributed to the presence of autoantibodies that result in the failure of synaptic signaling at the NMJ in muscle tissue. Failure of nerve impulse transmission may result from damaged, absent, or blocked AChR. Laboratory tests may be conducted to evaluate the presence of binding, modulating, or blocking AChR autoantibodies. A positive test result for the presence of AChR antibodies would be indicative of a likely diagnosis of MG.

Historically, laboratories have used radiolabeling as the gold-standard method for detecting blocking AChR antibodies.

**Table 2** Summary of Acetylcholine Receptor Blocking Results

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No.</th>
<th>Correlation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy donor</td>
<td>120</td>
<td>100</td>
</tr>
<tr>
<td>RIA screened positive</td>
<td>101</td>
<td>91</td>
</tr>
<tr>
<td>RIA screened negative</td>
<td>102</td>
<td>99</td>
</tr>
<tr>
<td>Total</td>
<td>323</td>
<td>97</td>
</tr>
</tbody>
</table>

RIA, radioimmunoassay.

*a*Shows correlation of radioassay with flow cytometric assay separated by clinical subtype.

**Table 1** Summary of Discrepant Results Including Binding, Modulating, and Blocking Results by Both Radioassay and Flow Cytometry

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age, y</th>
<th>AChR Binding</th>
<th>AChR Modulating</th>
<th>AChR Blocking by RIA</th>
<th>AChR Blocking by Flow Cytometry</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>52</td>
<td>Negative</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>M</td>
<td>52</td>
<td>Negative</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>F</td>
<td>80</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>M</td>
<td>84</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>M</td>
<td>62</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>F</td>
<td>96</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>F</td>
<td>47</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>M</td>
<td>60</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>M</td>
<td>83</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>M</td>
<td>66</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
</tr>
</tbody>
</table>

AChR, acetylcholine receptor; RIA, radioimmunoassay.

*The overall result correlation between these two methods is 97%. The results in this table represent the 3% of discordant results. When comparing the blocking results with the binding and modulating results, three of these result sets agree with the flow assay, four of these results agree with the radioassay, and the remaining three samples have results that are discordant across all antibody types.
as this test has been sensitive and reproducible. Several drawbacks, however, including cost associated with producing radiolabels, environmental and personnel hazards, and the generation of radioactive waste, have prompted the investigation into newer technologies for a suitable replacement for the current laboratory practices.

An AChR blocking antibody flow cytometric assay was designed here, and its performance was evaluated in comparison to current laboratory standards. The flow blocking assay was equivalent to the current RIA in its ability to detect AChR blocking antibodies. While both assays are clinically comparable, the flow assay offers advantages over radioassay, including enhanced control of QC and the use of a fluorescent label vs a radiolabel, which benefits both the laboratory and the environment.

The new flow cytometric assay measures a fluorescent label attached to the AChR receptor embedded in the surface of each cell. This distinguishing feature of the flow assay provides the opportunity to monitor cell receptor expression and cell counts along with measuring fluorescence. These characteristics of the flow cytometric assay allow for improved quality control compared with the RIA.

The 96-well format of the flow assay increases throughput, which can potentially decrease the turnaround time of patient results. In addition, the manufacturing and disposal of radioactive iodine is expensive and carries a greater environmental impact compared with the fluorescent markers used in the new assay. Laboratory personnel are also not exposed to radioactive substances when performing the flow assay, which decreases associated training and safety monitoring.

**Summary**

In summary, our research describes a new fluorescent flow cytometric AChR blocking antibody assay that is comparable in both sensitivity and specificity compared with the RIA. This assay has a high throughput, is cost effective, and is a more ecologically sound and efficient test.

**References**