Detection of Acetylcholine Receptor Modulating Antibodies by Flow Cytometry

Bucky K. Lozier,1 Thomas R. Haven, PhD,1,2 Mark E. Astill, MS,2 and Harry R. Hill, MD1-4

From the 1Department of Pathology, University of Utah School of Medicine, Salt Lake City; 2ARUP Institute for Clinical and Experimental Pathology, Salt Lake City, UT; 3Department of Pediatrics, University of Utah School of Medicine, Salt Lake City; and 4Department of Medicine, University of Utah School of Medicine, Salt Lake City.

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ABSTRACT

Objectives: To determine the clinical utility and performance characteristics of a laboratory-adapted flow cytometric method for the detection of acetylcholine receptor (AChR) modulating antibodies in myasthenia gravis (MG).

Methods: Serum samples from 120 healthy donors and 100 patients with suspected MG were assessed for the ability to reduce surface AChR concentrations (antigenic modulation) in RD (TE671) or DB40 human muscle cell lines by flow cytometry. Reference ranges were established by receiver operating characteristic curve analysis, and results were then compared with those of the current radioimmunoassay (RIA).

Results: Flow cytometric results from the RD cell line had an interpretive threshold of 46% modulation or greater and correlated best (98% sensitivity, 99% specificity) with those of the current radioimmunoassay (RIA).

Conclusions: The new flow cytometric method using the RD cell platform provided higher quality clinical results, a more robust and efficient assay format, a significant cost savings, and less environmental burden.

Myasthenia gravis (MG) is an autoimmune disease characterized by the presence of antibodies directed against acetylcholine receptors (AChRs) and occasionally other proteins at the neuromuscular junction in skeletal muscle. These antibodies cause failure of transmission of nerve impulses, resulting in progressive muscle weakness. Depending on epitope specificity, these antibodies can (1) bind and crosslink receptor subunits in such a way as to cause the receptors to be internalized and degraded in a process known as antigenic modulation, (2) functionally block the binding of the neurotransmitter acetylcholine (ACh), or (3) bind to and initiate complement fixation and cause disruption of the postsynaptic cell membrane.1

The disease may present clinically in two major ways. Ocular myasthenia is limited to the extraocular and levator palpebrae muscles, while generalized myasthenia is weakness that extends beyond the ocular muscles. The weakness is not present initially, and there is no generalized fatigue or pain, but muscle weakness becomes profound or almost paralytic...
after exertion. Symptoms of myasthenia most frequently initially manifest in relation to vision. Ptosis (paralytic closing of the eyelid), diplopia (double vision), and blurred vision are most commonly reported with ocular myasthenia, while generalized myasthenia can affect muscles of the oropharynx, extremities, and trunk. Involvement of the respiratory musculature occurs in approximately 15% of patients within 3 years of diagnosis. This respiratory paralysis, termed myasthenic crisis, may necessitate mechanical ventilation and plasma exchange, among other support measures.

Roughly 15% of patients with generalized myasthenia and 50% to 60% of patients with ocular myasthenia display no detectable levels of AChR antibodies and are classified as having seronegative MG. In patients who do display AChR antibodies, it has been shown that AChR modulating antibodies correlate best with clinical symptoms.

AChR modulating antibodies are currently detected in the clinical laboratory by using the rhabdomyosarcoma-derived TE671 (RD) cell line, an adherent cell that expresses fetal AChRs. Cultured RD cell AChRs are labeled with 125I α-bungarotoxin, and the cells are exposed to patient serum. In the presence of AChR modulating antibodies, radiolabeled receptors will be internalized, degraded, and expelled into the cell culture medium. The ratio of radioactivity in the culture supernatant vs total radioactivity can be used to determine percent receptor modulation.

Keefe et al demonstrated the ability to detect AChR modulating antibodies via flow cytometry using the DB40 cell line. The DB40 cell line is derived from the TE671 cell but has been transfected with genes for the stable expression of fetal and adult AChRs. This method, which uses fluorescent labels rather than radiolabels, would likely be of utility if adapted to the clinical laboratory.

The variability of symptoms at onset, heterogeneity of patient autoantibody profiles, and potential for life-threatening complications necessitate the best possible laboratory testing to aid in diagnosis. In this study, results from the detection of AChR modulating antibodies by flow cytometry using both the RD and DB40 cell lines were compared with results of the current live-cell–based radioimmunoassay (RIA).

Materials and Methods

Specimens

Serum samples from 120 healthy individuals were collected in accordance with University of Utah Institutional Review Board Protocol 7740. Healthy donors were screened via questionnaire regarding current state of health and presence or history of neurologic symptoms. Those with illness or a history of illness were excluded.

One hundred samples submitted to ARUP Laboratories for AChR modulating antibody testing were deidentified and tested in accordance with University of Utah Institutional Review Board Protocol 7275. Fifty samples were selected that showed positive results for AChR binding, modulating, and blocking antibodies by RIA, and 50 samples were selected that were AChR antibody negative. Clinical presentation and final diagnosis of patients with myasthenia were unavailable. Specimens were stored at –80°C indefinitely and up to 7 days at 4°C during testing.

Flow Cytometric AChR Modulating Antibody Detection Assay

Samples were assayed on both the RD cell line (CCL-136; ATCC, Manassas, VA) and the DB40 cell line (David Beeson, PhD, and Isis Innovation, Oxford, UK) for the presence of AChR modulating antibodies. 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 (Salt Lake City, UT) and consisted of Eagle’s minimum essential medium (EMEM) supplemented with 10% heat-inactivated Fetal Clone III, minimum essential medium (MEM) essential and nonessential amino acids, and MEM vitamins. Culture medium for DB40 cells was supplemented with 0.5 mg/mL geneticin (Gibco, Grand Island, NY). Cells were harvested from flasks using 1× phosphate-buffered saline (PBS) containing 9 mmol/L EDTA, washed, and resuspended in EMEM, and 100 µL of cell suspension was dispensed into a 96-well tissue culture plate at a concentration of 1 × 10⁵ cells per well. Cells to serve as background secondary antibody or maximum receptor staining standards were exposed to an additional 4 µL of cell culture medium (no serum exposure). Then, 4 µL of control or test serum samples was then introduced to the cell culture medium of each remaining well (1:26 dilution), and the plate was incubated overnight at 37°C, in 5% CO₂, and at 95% humidity to allow for AChR modulation. Following the overnight incubation to allow for AChR modulation, plates were decanted to remove patient and control dilutions and then inverted and gently blotted. Next, 100 µL of assay buffer (0.5% bovine serum albumin in Hank’s balanced salt solution without Ca²⁺ or Mg²⁺) was added to background stain wells, followed by addition of 100 µL of 2 µg/mL mAb 210 (Abcam, Cambridge, MA), a monoclonal antibody specific for the main immunogenic region (MIR) of human AChR α1 subunits, in assay buffer to all remaining control and patient wells. Cells were incubated for 30 minutes in the dark at room temperature and then gently washed twice with 200 µL of assay buffer. Plates were then inverted and blotted. All cells were then stained with 100 µL of 4 µg/mL APC-conjugated goat anti–rat IgG (BD Biosciences, San Jose, CA) for 30 minutes in the dark at room temperature. Cells were again washed, and 100 µL of 9
mmol/L EDTA-PBS was added and allowed to incubate for 10 minutes in the dark at room temperature. Cells were then dissociated by pipetting, followed by the addition of 50 µL each of assay buffer and 1% paraformaldehyde in PBS. The fixed cell suspension was then transferred to cytometer tubes and analyzed on a BD FACSCanto II cytometer using Diva software (BD Biosciences).

No viability staining was used prior to paraformaldehyde fixation. In past experiences, nonviable cells were observed via trypan blue dye exclusion to dissociate freely. It was assumed nonviable cells would be washed away prior to completion of the staining procedure, and any cells present at the end of this procedure would be adherent and therefore viable. Initially, the fluorochrome FITC was used. In an attempt to minimize innate cellular fluorescence, APC was selected based on difference in emission spectra. No other fluorochromes were investigated since APC seemed to work well.

**Data Analysis**

Results were initially calculated separately and reference ranges established based on (1) mean fluorescence of receptor staining corrected for innate cellular and nonspecific fluorescence, (2) mean fluorescence of the total population, and (3) percentage of the population above background fluorescence. Results from the first calculation strategy compared most favorably with those of the current RIA assay and are included in this article.

Cell populations were gated using forward scatter/side scatter characteristics to eliminate cell fragments and aggregates. Cursor gates were applied to the histogram to discriminate between innate cellular fluorescence and nonspecific or receptor-associated staining fluorescence. AChR percent modulation was calculated by comparing the mean geometric fluorescence of receptor-associated staining with the maximally stained assay standard using the following equation:

$$\frac{1 - [(\text{Sample} - \text{Nonspecific})]}{(\text{Maximum} - \text{Nonspecific})]} \times 100\%$$

The assay reference range was established by receiver operating characteristic (ROC) curve analysis of flow cytometric vs current assay results. Area under the ROC curve (AUC) was evaluated as an indicator of assay performance. Software used for the ROC curve analysis was SAS version 9.1 (SAS Institute, Cary, NC). Results of the new flow cytometric assay were then evaluated using those of the current AChR modulating antibody RIA as the standard for assessment of performance.

**Results**

All histograms displayed unimodal fluorescence distribution. **Figure 1** Nonspecific (background) stained assay standard showing innate cellular fluorescence (P2). APC-A, allophycocyanin; FSC-A, forward scatter; SSC-A, side scatter.

**Figure 2** Maximally stained assay standard showing receptor-associated fluorescence (P3). APC-A, allophycocyanin; FSC-A, forward scatter; SSC-A, side scatter.
illustrate examples of gating and histogram results for assay standards and controls. The ROC curve plot in Figure 5 shows an AUC value of 1.00, indicating excellent sensitivity and specificity for adherent RD cell AChR modulating antibody results.

Figure 6 illustrates AChR modulating antibody result distribution for the RD cell line calculated using mean population fluorescence after background staining normalization. Positive cutoff at 46% modulation (n = 220). Area under ROC curve = 1.0000.

AChR modulating antibody-negative sample results display little to no receptor modulation (≤40%), while the majority of the positive samples display high to complete receptor modulation. This separation of result populations suggests a good dynamic range for the assay with less potential for false-negative or false-positive results.

The linear regression plot in Figure 7 shows good result correlation (R² = 0.84) between results for flow cytometric RD cell modulating results and those of the current RIA assay for modulating activity. The ROC curve plot in Figure 8 shows an AUC value of 0.98, indicating very good sensitivity and specificity for DB40 cell AChR modulating antibody results.

Figure 9 illustrates AChR modulating antibody result distribution for the DB40 cell line calculated using mean population fluorescence after background staining normalization. Positive cutoff for this calculation scheme was determined by ROC analysis to be 25% modulation or greater. Results for healthy donor and AChR modulating antibody-negative samples show little to no receptor modulation, while most positive results show a high (>70%) level of modulation. Only three expected positive samples (positive by RIA) resulted as negative in relation to the current assay using the DB40 cell line.

The linear regression plot in Figure 10 shows reasonable result correlation (R² = 0.73) between results for flow cytometric DB40 cell modulating results and those of the current RIA assay for modulating activity. Comparison of the current RIA qualitative results vs AChR modulating
The DB40 cell line demonstrates slightly lower assay performance than the RD cell line, possibly due to defects in receptor turnover or metabolism subsequent to gene transfection. Both cell lines demonstrated adequate receptor staining in the assay standards, but several of the AChR modulating antibody-positive samples did not elicit a significant decrease in receptor expression when exposed to the DB40 cells. Both cell lines tolerated the assay procedure with minimal sloughing and good cell recovery for flow cytometric analysis. The maximally stained assay standard provides an objective measure of cell performance with regard to receptor expression. This measure of receptor expression allows for anticipation of cell line failure and avoidance of delays in testing.

**Discussion**

Detection of AChR modulating antibodies by flow cytometry is a viable alternative to the current RIA. Results from the RD cell line correlate best with the current RIA when calculated using mean population fluorescence.
attenuated for nonspecific secondary antibody staining and inherent cell fluorescence. Two of the 220 results for the RD cell flow cytometric assay were discrepant with those of the current RIA. These included one normal donor sample positive by RIA and negative by flow (expected negative), the other an AChR binding and blocking antibody-positive sample negative by RIA and positive by flow (expected positive). On the basis of these results, the flow assay is more reliable and exceeds performance of the current RIA. In all, three expected positive samples yielded negative results using the DB40 cell line, but only two samples had discrepant results using the RD cell line.

Culture and assay conditions for the RD and DB40 cell lines in this study were identical except for addition of 0.5 mg/mL geneticin (G418) to the DB40 cell culture medium, which was necessary to maintain the transfected cell line. It is possible that the presence of G418 is responsible for differences in AChR modulation results between the RD and DB40 cells but not likely due to the random and scant occurrence of discrepant results.

Compared with the current RIA, sensitivity and specificity of the DB40 cell line were 94% and 98%, respectively. The RD cell line showed 98% sensitivity and 99% specificity. Based on these data, and when taking into account increased costs associated with acquisition and maintenance of the DB40 cell line, use of the RD cell line for this flow cytometric AChR modulating antibody assay seems warranted.

This new assay format not only exceeds performance of the current RIA assay but also allows for the quantification of receptor expression on the RD cell, facilitating more objective measurement of assay performance. Using this receptor quantification, our laboratory has been able to track cell culture age-related changes in receptor expression and replace cell cultures before we experience failure of the clinical assay. This enhanced assay performance monitoring has led to fewer delays in reporting clinical results due to cell culture failure, a drawback not uncommon to the current RIA method.

One last advantage of the new assay format is the ability to truly differentiate AChR modulating from AChR

![Figure 9](image-url) Flow cytometric result distribution for adherent DB40 cells calculated using mean fluorescence of gated cells after background fluorescence normalization. Ab, antibody; AChR, acetylcholine receptor; Mod, modulating; Neg, negative; Pos, positive.

![Figure 10](image-url) Adherent DB40 cell results correlation with the current assay. Results calculated using mean fluorescence of gated cells after background fluorescence normalization. y = 1.3349x + 6.9582. $R^2 = 0.73$. AChR, acetylcholine receptor; RIA, radioimmunoassay.
blocking antibodies. Both modulating and blocking antibody RIA assay formats currently rely on $^{125}$I α-bungarotoxin to label and quantify AChRs. AChR antibodies show a dissociation constant ($K_D$) of $2.35 \times 10^{-11}$ mol/L, while α-bungarotoxin has a $K_D$ of $2.6 \times 10^{-10}$ mol/L. Therefore, AChR autoantibodies specific for the AChR ACh binding site may displace the radiolabeled bungarotoxin, leading to a falsely elevated modulating antibody result.\textsuperscript{11} By labeling the MIR with a monoclonal antibody rather than the ACh binding site, there is no opportunity for the radiolabel to be displaced by a nonmodulating autoantibody, and thus one potential source of erroneously elevated AChR modulating antibody result is eliminated.

Here, we demonstrate the feasibility, reliability, and improved safety of a fluorescent AChR modulating antibody assay employing RD cells in the evaluation of patient serum samples suspected of having MG. There is a 57% cost savings with the new method due to reagents, materials, technician time, and waste disposal. Due to the greater simplicity of the new method, training poses no problems, assay troubleshooting is simplified due to the ability to quantify receptor expression, and sample requirements are identical between the two methods. This assay appears to have several significant advantages to that of the radiolabeled receptor modulation assay.

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Address reprint requests to Dr Hill: University of Utah School of Medicine, 50 North Medical Dr, Rm 5B114, Salt Lake City, UT 84132; Harry.Hill@path.utah.edu.

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References