DEVELOPMENT OF AN ASSAY FOR THE DETECTION

OF ACETYLCHOLINE RECEPTOR BLOCKING

ANTIBODIES BY FLOW CYTOMETRY

by

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STATEMENT OF THESIS APPROVAL

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ABSTRACT

Myasthenia Gravis (MG) is a neuromuscular autoimmune disease primarily

associated with the presence of antibodies directed toward receptors embedded in the

motor end-plate at the neuromuscular junction (NMJ). These antibodies cause blockage,

dysfunction, and degradation of acetylcholine receptors (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.

Detection of acetylcholine receptor antibodies through the use of a radio-label has

become standard procedure in most laboratories. Known drawbacks are associated with

radioimmunoassay; cost of radioisotopes, hazards to laboratory professionals, and

manufacture and disposal of radioactive materials have prompted investigation into

replacement assays.

In 2009, scientists described a method of detecting AChR modulating antibodies

using flow cytometric techniques. They utilized fluorescently-labeled molecules to aid in

the detection of modulated AChR and to confirm the presence of AChR modulating

antibodies. We postulate that similar techniques could enable the conversion of the AChR

blocking assay to flow cytometry as well.

Described here is a high-throughput immunofluorescent flow cytometric assay

designed for the detection of AChR blocking antibodies. Three-hundred-twenty-four

sera were tested on both the AChR blocking radio-assay and the new immuno-

fluorescent flow cytometric assay. Analysis of the results revealed a 96.9%

concordance between the two assay methodologies. 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%)

when compared to radio-assay.

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INTRODUCTION

Myasthenia Gravis (MG) is a neuromuscular, autoimmune disorder which leads to skeletal muscle weakness and fatigability. This muscle fatigability is a distinguishing symptom of MG as it is exacerbated after periods of activity and is reduced after periods of rest. Another hallmark of this disorder, as in contrast to other neuromuscular disorders (i.e., Congenital Myasthenic Syndrome, or Lambert-Eaton Syndrome), is the involvement of ocular muscles either during the initial onset of symptoms or sometime during the course of the disease (1, 2, 3).

Symptoms of MG may appear suddenly or present intermittently. Muscle weakness ranges from mild to severe, and the weakness can either be localized, particularly when the eye or facial muscles are involved, or generalized, thereby affecting many muscle groups. A particularly dangerous clinical presentation of MG involves weakness in the muscles responsible for the control of breathing.

A life-threatening complication of MG, called myasthenic crisis, is of particular concern for patients who already experience respiratory muscle weakness. Myasthenic crisis is a paralysis of the respiratory muscles, and patient survival requires that breathing be sustained by a mechanical ventilator. Crises may be triggered by emotional stress, infection, or even adverse reactions to medication (4).

The thymus gland is found to be abnormal in roughly 75% of MG patients (5, 6). Abnormalities include thymic hypertrophy and thymoma, which is either a benign or

malignant tumor of the thymus. Benign tumors may become malignant, and malignant thymomas may spread to other tissues or can potentially cause other types of disease.

This disease is considered rare with a prevalence estimated to be 64-90 cases per 1,000,000 people. It exhibits bimodal onset peaks, with the peak onset being between the ages of 20-30 for women, and, for men, between the ages of 55-60. Aside from these peak populations, MG can affect people of all ages, ethnicities, and of either gender (7). Genetic predispositions, however, have been noted, and factors such as a personal or family history of other autoimmune disorders, or HLA type B8, DR1, and DR3, are thought to play a role in the likelihood that an individual may develop this disease (8).

Understanding MG and how it is diagnosed require knowledge of the immune system, dynamic interactions that occur in the neuromuscular junction, physiology of the acetylcholine receptor (AChR), effector mechanisms of MG AChR associated antibodies, and laboratory techniques used for antibody detection.

<u>Immune System Function</u>

The immune system is comprised of a set of biological structures and interdependent processes designed to protect the body against disease. Diverse functions of the immune system include pathogen detection, coordinated attack on invading agents ranging from viruses to parasites, and the ability to distinguish foreign invaders from the organism's own healthy tissue.

The thymus gland is an important component of the immune system and is integral in the development of immunity in early life. In infants, a normal thymus gland is relatively large, and it continues to grow until puberty. After puberty, this gland begins to

atrophy and by adulthood is largely replaced by adipose tissue; nevertheless, effects of thymus function are retained through adulthood (9, 10). In MG patients, the thymus gland frequently remains large and abnormal (5, 6).

The thymus is a specialized gland responsible for the maturation of hematopoietic progenitor cells into T-lymphocytes. Commonly known as T-cells, T-lymphocytes are a type of white blood cell that is a fundamental component of the adaptive immune system. T-lymphocytes can be distinguished from other types of lymphocytes, namely B-cells and NK-cells, due to the presence of specialized receptors that are embedded in the T-cell membrane (11).

T-cell receptors (TCR) are highly diversified, and the large assortment of TCR translates into the ability of the immune system to identify and attack a vast array of targets. The random nature of gene-segment shuffling in TCR results in the creation of antibodies directed toward normal host molecules. In fact, one of the primary functions of the thymus is to recognize and inactivate T-cells which have formed to attack normal body proteins and healthy tissues. This recognition and elimination process is known as central tolerance and is intended to reduce autoimmunity (12).

Autoimmune disorders are caused by a flaw or defect in this recognition process which creates an aberrant immune system. This faulty immune system produces a misguided immune response leading to the body's inability to distinguish between foreign invaders and healthy body tissues and may result in an attack on otherwise normal proteins, cells, or organs.

Inappropriate autoimmune responses cause a wide variety of diseases such as Systemic Lupus Erythematosus, Rheumatoid Arthritis, Addison's disease, Celiac disease,

and MG. The symptoms of distinct autoimmune conditions are equally diverse and can affect any area of the body. MG patients often experience muscle weakness that can be attributed to auto-antibodies directed toward the AChR in muscle cells (1, 2, 3).

Neuromuscular Junction

The neuromuscular junction (NMJ) is the physiological structure where the neuron activates muscle contraction by transmission of excitatory signals (or action potentials) from the central nervous system via the neurotransmitter, acetylcholine. The two main functional units of the NMJ are the axon terminal of a motor neuron which is filled with vesicles containing acetylcholine, and the motor end-plate of the muscle cell which expresses AChR in the membrane. The axon terminal and the motor end-plate have individual plasma membranes which are separated by a 50 nm gap known as the synaptic cleft (13).

The AChR are ligand-gated ion channels which open when acetylcholine crosses the synaptic cleft and binds to them. This binding leads to the production of an electrochemical gradient that produces a localized depolarization of the motor end-plate leading to muscle contraction.

Interference in the transmission of the signal across the synaptic cleft in the NMJ can result in a mild to complete loss of muscle function. A signal transmission can be blocked or decreased by chemicals found in medications, by toxins (such as α-Bungarotoxin, a neurotoxin isolated from snake venom), or by the onset of disease states such as MG (14).

Acetylcholine Receptor

The AChR, an integral membrane protein found on the motor end-plate of muscle cells, is a pentameric ligand-gated ion channel. The five subunits are arranged to form a central pore which has the ability to open and close with a twisting motion to allow the flow of ions through the muscle cell membrane. The AChR can be expressed in an embryonic form, where the five subunits are composed of two units of α, and one β, δ, and γ subunit, or an adult form composed of two units of α, and one β, δ, and ε subunit (15).

Each of the five subunits is constructed similarly of four distinct domains. Of particular interest is the N-termini domain which is located on the extracellular membrane surface. Acetylcholine, when present in the synaptic cleft, can bind to the N-termini of each of the two subunits of α, which results in a conformational change causing the subunits to rotate and open the central pore (15). The open pore allows Na^+ to enter the cell from the synaptic cleft causing depolarization and the generation of an end-plate potential which signals the muscle to contract.

Other molecules, aside from acetylcholine, also have the ability to bind to AChR. These other molecules, for example, curare, hexamethonium, and α-Bungarotoxin, bind to the N-termini of the α subunit(s) but do not cause the same conformational change which opens the pore (13, 14). The binding of these interfering substances disrupts the normal action of acetylcholine and blocks the signal in the NMJ thereby obstructing the signals for muscle contraction. The muscle weakness, as experienced by MG patients, is frequently caused by the presence of autoimmune antibodies which act as interfering molecules and bind to the AChR, inhibiting the normal action of acetylcholine (16).

Acetylcholine Receptor Antibodies

Auto-antibodies with affinity for AChR encompass the majority of MG associated antibodies. These antibodies cause blockage, dysfunction, and degradation of AChR leading to muscle weakness and fatigability. Three main types of AChR antibodies have been identified and are known as binding, modulating, and blocking.

Each of these three antibodies has a different effector mechanism. The binding AChR antibody causes binding and activation of complement at the NMJ. When the binding antibody forms a complex with the AChR (antigen), the complex is recognized by the complement system which proceeds to attack the receptor and causes permanent destruction of AChR (17, 18, 19).

Modulating AChR antibodies cause accelerated degradation of AChR molecules that have been cross-linked by the antibody. This is also referred to as antigenic modulation. Cross-linking renders the receptor useless, subsequently, the receptor is endocytosed and degraded. The presence of this specific antibody is indicative of a more severe form of the disease (19).

The AChR blocking antibody causes a functional blockage and impairs acetylcholine signaling due to antibody binding at the normal ligand binding site on the α subunit (20). This mechanism of AChR disruption is less common 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 (21). Studies have shown that a correlation exists between the level of blocking antibody detected in the patient serum and the severity of generalized muscle weakness experienced (18, 19).

Diagnosing Myasthenia Gravis

Diagnosing MG can be a difficult task, as the symptoms may be subtle and difficult to distinguish from those of other disorders. Clinicians have a variety of tests available to aid them in making a diagnosis. The two major categories of diagnostic tools available are nonserological type tests, and serological testing.

Examples of nonserological tests include a thorough physical examination, muscle fatigue testing, ice tests, electrodiagnostics, imaging studies, pulmonary function evaluations, and administration of the edrophonium test.

Serological tests to identify the presence of AChR antibodies associated with MG symptoms can be performed in the clinical laboratory and include radioimmunoassay (RIA) and flow cytometry (22, 23, 24).

Radioimmunoassay

Most of the MG serological tests are conducted by RIA with the exception of the AChR modulating antibody which can also be tested for by flow cytometry at Associated Regional and University Pathologists Laboratories (ARUP) in Salt Lake City, UT. The general concept behind RIA in its use for detecting AChR antibodies is that the presence of an antibody in patient serum can be semiquantitatively detected by adding a known quantity of radioactively labeled molecules to AChR after exposing the AChR to patient serum (22, 23).

The radiolabeled molecule and the AChR blocking antibody compete for binding sites on the AChR. The radiolabeled molecule can only bind to the receptor site when an

antibody, if it is present in the patient serum, has not already bound to it. Therefore, radioactivity can only be detected when the antibody is not present in the patient serum.

As a first step in performing this assay, human cells that express AChR are grown in the laboratory and harvested. The receptors embedded in these cells act as a substitute for the receptors found at the motor end-plate of the patient muscle cells. Blood is drawn from the patient, and the serum is separated from the red blood cells. Serum from the patient is then added to the laboratory-grown cells, and, if the AChR antibodies are present in the patient serum, they will normally bind to the receptor site. After allowing time for the antibodies to bind, the cells are washed to remove any potentially interfering substances from the reaction mixture.

After the cells are washed, the receptors will either have antibody bound from the patient serum, or they will remain unbound. An iodine-125 radiolabeled molecule is subsequently added to the cells which will bind to any open receptor sites. In testing for AChR blocking antibodies, the radiolabeled molecule used is α-Bungarotoxin.

α-Bungarotoxin is a neurotoxin isolated from snake venom and is one of the substances that readily binds to the α subunits of the AChR (14). If AChR blocking antibodies are present the α-Bungarotoxin will not bind as the receptor site will already be occupied. After the addition and incubation of α-Bungarotoxin, the cells are again washed, and any unbound substances are removed.

The cell preparations are then placed in a gamma counter, and the radioactivity of the sample is then detected and measured. The radioactive signal is inversely proportional to the quantity of bound antibody; hence, a low radioactive signal indicates the patient

has detectable AChR antibodies. Conversely, a high radioactive signal indicates no antibodies are bound to the AChR, and the assay result is negative.

While RIA testing is fairly sensitive and reliable for the patient, it is unfortunately a process that poses a health risk for the personnel who work in the laboratory and who are repeatedly exposed to radioactive material. The environment is also negatively impacted by the manufacture and disposal of radioactive waste. With this awareness, laboratories are working toward creating other assays to protect their laboratory staff.

Flow Cytometry

Flow cytometry is a technology that counts and analyzes particles such as cells. Multiple physical characteristics of single particles including relative granularity, internal complexity, and relative fluorescence intensity are recorded with this instrument. The multiparametric analysis of characteristics is conducted by suspending particles in a stream of fluid and passing them through a laser-based electronic detection apparatus. These instruments can count and analyze thousands of particles per second.

In 2009, scientists described a method of detecting AChR modulating antibodies using flow cytometric techniques (24). They utilized fluorescently-labeled molecules to aid in the detection of modulated AChR and to confirm the presence of AChR modulating antibodies.

That publication inspired the development of a flow cytometric assay to replace the RIA assay for the detection of AChR modulating antibodies at ARUP (25). This new flow cytometric assay went into production in 2012 and has entirely supplanted the RIA technique for detection of this specific antibody.

We postulate that this development could also support the conversion of the AChR blocking antibody assay to flow cytometry as well. This new assay would be performed similarly to RIA; however, a fluorescently labeled α-Bungarotoxin would be used instead of an iodine-125 radiolabeled α-Bungarotoxin. The fluorescence, rather than the radioactivity, is detected and measured through a flow cell with the use of laser technology, eliminating the radioactivity danger imposed by the RIA testing process.

MATERIALS AND METHODS

The rhabdomyosarcoma (RD) cells used for this research were obtained from American Type Culture Collection, Catalog Number CCL 136 RD (Manasses, VA). The DB40 cell line used was, Catalog Number 3601c, Isis (Oxford, UK). The fluorescent label for this assay was α-Bungarotoxin Alexa Fluor® 647 Conjugate 500 μg/mL, Catalog Number B35450, from Molecular Probes (Grand Island, NY). G-418, Geneticin® was added to the cell culture medium for the DB40 cell line only, Catalog Number 10131035, from Gibco (Grand Island, NY).

Other reagents required for this assay were Hank's Balanced Salt Solution (HBSS) without calcium, magnesium, or phenol red, Catalog Number 45000-464, from VWR (Radnor, PA), Bovine Serum Albumin (BSA), Fraction V, Catalog Number A2153, from Sigma-Aldrich (St. Louis, MO). The Phosphate Buffered Saline (PBS), Ethylene-Diaminetetraacetic Acid (EDTA), and Paraformaldehyde 1% in PBS were all supplied by ARUP Laboratories Reagent Lab (Salt Lake City, UT).

Eagles Minimal Essential Medium (EMEM), cell culture reagent, was produced by the ARUP Laboratories Reagent Lab and consisted of EMEM supplemented with heat-inactivated Fetal Clone III, MEM essential and nonessential amino acids, antibiotics, and MEM vitamins. Serum control lots were prepared by ARUP Reagent Lab, Catalog Number A-00499, and included a Negative Control, a Positive-I Control (low positive), and a Positive-II Control (high positive).

Preparation of Cells

The anchorage-dependent RD cells were grown in T-175 tissue culture flasks in a

37°C, 5% CO_2, 95% humidity incubator using 10% EMEM growth medium. Confluent

monolayers were observed under a microscope approximately seven days after seeding

the flasks. The cells were then released from the flask surface by decanting the growth

medium and adding PBS with EDTA. After 10 minutes of incubation at 37 °C the cells

were detached and the cell suspension was removed, placed in a 50 mL conical tube, and

centrifuged for 5 minutes at 1500 xg.

After centrifugation the cells were re-suspended and counted using a Coulter

counter and a fraction was introduced back into the T-175 flask to continue serial

propagation. The remaining cells were suspended in 10% EMEM at a cell suspension of

1 X 10^6 cells/mL and were then vortexed and used for the flow cytometric assay.

The preparation and propagation of DB40 were much the same as that of the RD

cells. The distinguishing step was the addition of 0.5 mg/mL Geneticin® to the 10%

EMEM cell medium as DB40 cells are transfected with cDNA which encodes the β, δ,

and ε subunits of the AChR (26). The DB40 cell line expresses both the embryonic and

adult forms of AChR, whereas the RD cell line expresses the embryonic form of AChR

with the γ subunit.

Control Preparation

The Negative Control was composed of pooled, normal human serum. The

Positive-I Control was manufactured by the ARUP Reagent Lab by pooling patient sera

with low-positive AChR blocking results. The Positive-II Control is made by pooling

patient sera with high-positive AChR blocking results. All controls are filtered and aliquoted into sterile microcentrifuge tubes and kept at -70°C for long-term storage. Thawed controls are stable for one week at 2-8°C.

These three control levels are used for quality assurance purposes. The expectation is that the Negative Control result should be negative on every run. An elevated Negative Control result could signal possible false positive results on a run, and, in this event, the samples with positive results would be repeated. Positive-I Control results should be in the low positive range, while Positive-II Control results should be in the high positive range. Positive control values that fall outside the established range would consequently indicate assay performance issues for that run and require repeat testing of samples.

Flow Cytometric Assay Protocol

The flow cytometric AChR blocking antibody assay was performed in a 96 well flat-bottom tissue culture plate by adding 100 μL cells suspended at 1 X 10^6 cells/mL to each well. The 96 well plate was gently agitated to evenly distribute the cells across the bottom. The plate was placed in a 37°C, 5% CO_2, 95% humidity incubator overnight for 12 to 24 hours to allow for cellular attachment to the plate bottom.

After this overnight incubation, the preparation for this assay was initiated by first bringing all reagents, including HBSS + .5% BSA, control sera, and patient samples to room temperature. Then 10 μL of appropriate specimen were added to each well following the map as illustrated in the template (Fig. 1). Assay buffer, 10 μL, was added to each of the background and maximum stain wells.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BG	BG	MAX	MAX	NEG (=)	LOW (+)	HIGH (++)	1	2	3	4	5
B	6	7	8	9	10	11	12	13	14	15	16	17
C	18	19	20	21	22	23	24	25	26	27	28	29
D	30	31	32	33	34	35	36	37	38	39	40	41
E	42	43	44	45	46	47	48	49	50	51	52	53
F	54	55	56	57	58	59	60	61	62	63	64	65
G	66	67	68	69	70	71	72	73	74	75	76	77
H	78	79	80	81	82	83	84	85	86	87	88	89

Figure 1. Template of 96 well plate set up. This template maps the location on the 96 well flat-bottom tissue culture plate where all controls and samples are to be added. This information should also be used when numbering the 12 x 75 mm flow cytometry tubes that these samples will be transferred into.

Background signal (BG) remains unstimulated by patient sera or fluorescent labels (Fig. 2). This BG represents the inherent low level fluorescence expressed by the cells and is plated in duplicate. The maximum signal (MAX) is stimulated by the fluorescent α-Bungarotoxin only and is not exposed to patient serum (Fig. 3). The MAX is also plated in duplicate. The BG and MAX geometric means are both used for final patient result calculations.

After adding $10 \mu L$ of appropriate specimen to each well as indicated by the template, the plate was gently agitated to mix specimens. The plate was incubated for 2 hours at room temperature. The growth medium was carefully decanted, the plate was inverted, and then lightly blotted. Cells anchored to the bottom of the plate were washed twice with $200 \mu L$ HBSS + .5% BSA assay buffer using a multichannel pipette. The buffer was dispensed slowly, high up on the sides of the wells where it ran down the side
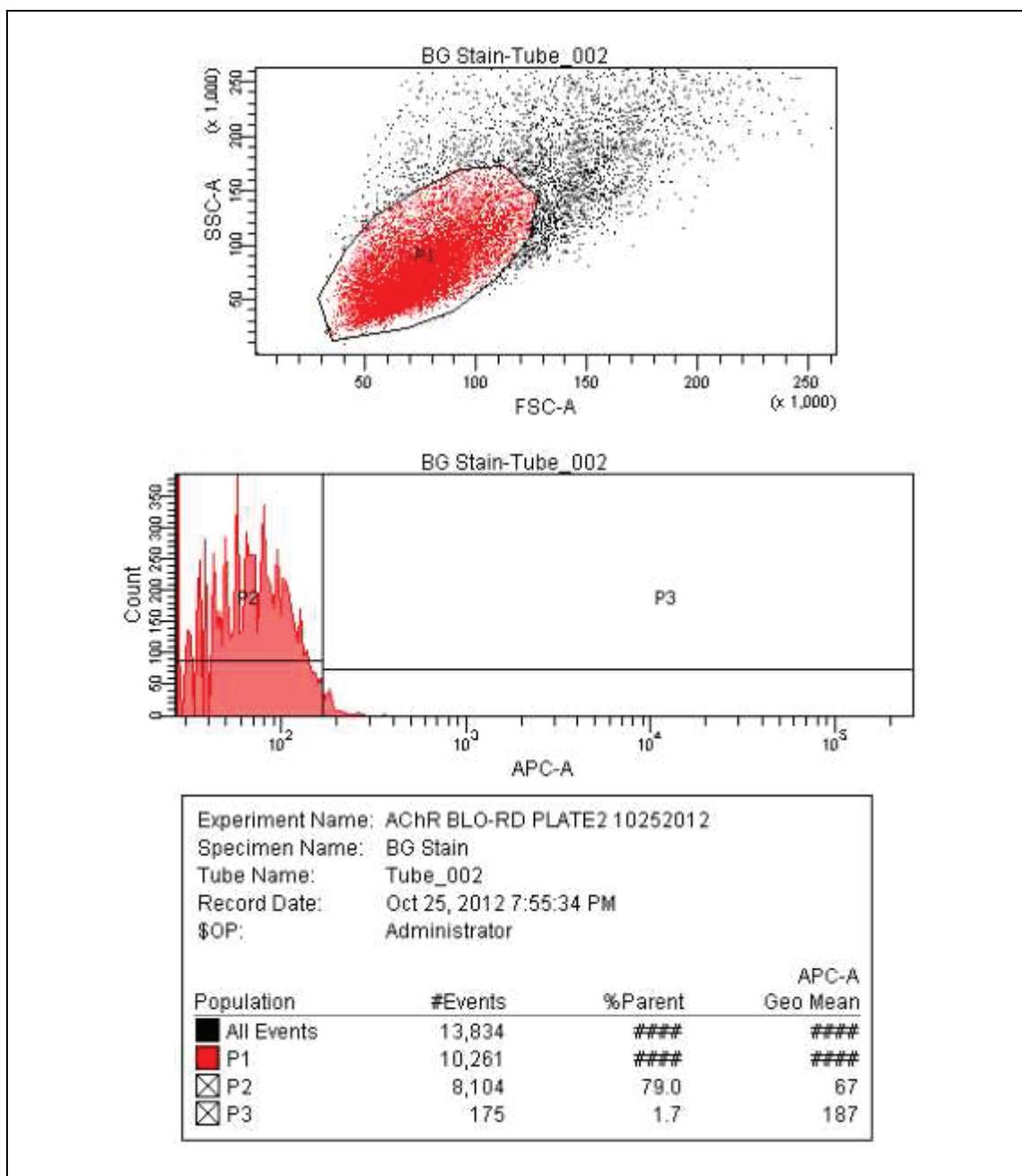
Figure 2. Flow plot of unstimulated background cells. The gated cell population P1 was set to exclude cell fragments and aggregates. The discriminator line was set so that the %Parent in P3 was less than 2%. The APC-A Geo Mean, or geometric mean, was averaged and the average result of the two BG stains was subtracted from all control and patient sample geometric means. If the CV% was high between the geometric mean values of both background tubes, borderline samples were repeated.
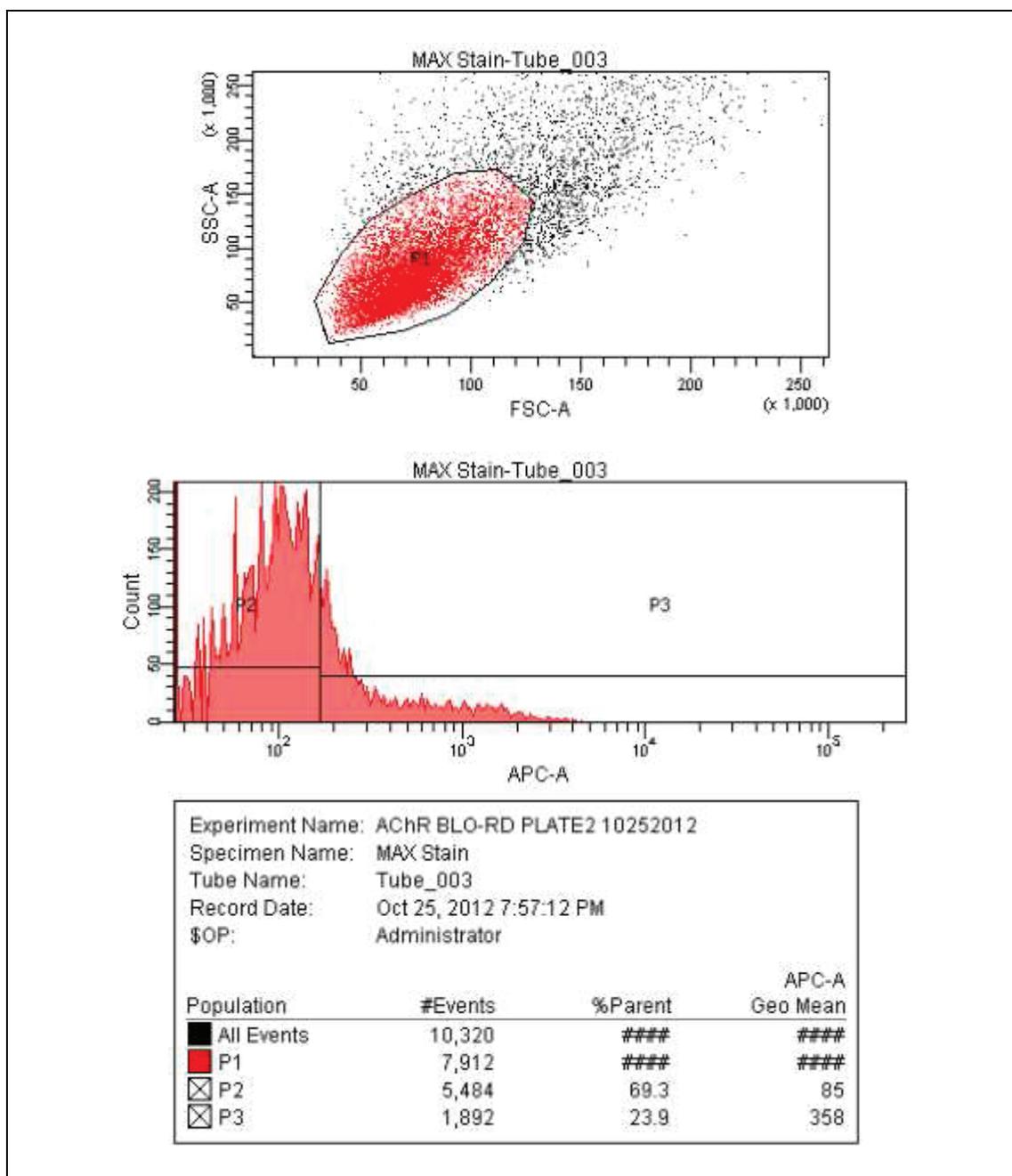
Figure 3. Flow plot of maximum stain cells. The maximum stain cells are stimulated with fluorescently labeled α-Bungarotoxin in the absence of patient serum. The geometric means of both MAX tubes are averaged and used for the calculation of control and patient sample results. A high CV% would indicate QC issues and the possible need for repeat testing.

of the well and into the bottom. The plate was decanted and blotted and this wash process was again repeated.

Assay buffer, 100 μL per well, was dispensed into all wells. An additional 10 μL of assay buffer was added to each of the two BG wells and 10 μL diluted α-Bungarotoxin Alexa Fluor® 647 Conjugate was added to each of the remaining wells. The plate was incubated for 60 minutes at room temperature in the dark to protect the fluorescent label from light.

Following this incubation the plate was decanted, blotted, and washed two times according to the wash protocol as stated above. The cells were then dissociated from the 96 well plate by adding 100 μL 9mM EDTA-PBS and incubating in the dark for 30 minutes. Using a multichannel pipette, 50 μL assay buffer and 50 μL 1% para-formaldehyde in PBS were added to each well. The contents of each well were vigorously pipetted in order to dissociate all cells.

The fixed cell suspension from each well was then transferred to a prelabeled, 12 x 75 mm flow cytometry tube. The sample preparations were protected from light by covering tubes with foil while waiting to be analyzed on the instrument. The tubes were then placed on a carousel and analyzed on a BD FACSCanto II cytometer using Diva software.

Fluorescent Measurement and Result Calculation

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

the 96 well plate. Both the background and maximum stain geometric means were used to calculate specimen results.

Cell population selection was made so that gate P1 excluded cell fragments and aggregates. The P2 gate discriminator line was set on the background so that less than 2% of fluorescence across the entire histogram is to the right of the discriminator line starting at channel 1 and ending on 10000 on a 1024 channel histogram (Fig. 2). A right shift of the peak into P3 indicates the presence of the fluorescent marker (Fig. 3).

The percent blocking result is defined as the change in proportion of fluorescent cells that are observed in the P3 gate relative to the background stain. The average geometric mean LFL1 value from the background stain is subtracted from all other LFL1 geometric mean readings. A high CV% across the two background wells would indicate 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 related to the result.

<u>Study Cohort</u>

Serum samples from 324 individuals were collected and tested. These samples were selected based on three categories including healthy donors, screened RIA AChR negatives, and screened RIA AChR positives. The healthy donor population consisted of 120 self-proclaimed healthy individuals, and sera were collected in accordance with University of Utah Institutional Review Board Protocol #7740.

The 204 disease population was composed of an RIA AChR blocking negative group and an RIA AChR positive group. These samples were submitted to ARUP Laboratories for AChR blocking antibody testing, were de-identified and tested in accordance with University of Utah Institutional Review Board Protocol #7275. We did not have access to the clinical history in these de-identified positive samples.

RESULTS

In this study, we assessed the performance characteristics of the newly designed flow cytometry based AChR blocking antibody assay relative to those of the currently employed AChR blocking antibody radioimmunoassay. Samples were assayed on both the RD cell line and the DB40 cell line for the presence of AChR blocking antibodies.

Reference Intervals

Threshold values were determined for both RD and DB40 cell lines on the flow cytometric assay. These reference intervals were calculated using Wilcoxon Signed Rank, Kruskal-Wallis, and Receiver Operator Curve statistical analyses. The positive threshold value for the RD cell line was calculated to be 37%; therefore, a result of greater than or equal to 37% is determined to be positive.

The positive threshold value for the DB40 Cell line was calculated to be 40%; therefore, a result of greater than or equal to 40% is considered to be positive. These cutoff values were used for determination of qualitative categorization and subsequent comparison with the current RIA assay results. The reportable range for this assay is 0% to 100% and any result of <0% is considered to be 0% and any result >100% is considered to be 100%.

<u>Self-Proclaimed Healthy Donors</u>

One-hundred-twenty self-proclaimed healthy donor sera were tested during this assay evaluation. Of the 120 samples, 119 produced negative results on the RD cell line. The remaining sample exhibited a low event count possibly due to the presence of an interfering substance. A given sample needed to have at least 3000 events recorded in order to be considered valid for this evaluation. Upon repeat testing the same phenomenon was observed. More testing is required to determine the significance of low event counts.

All 120 self-proclaimed healthy donor samples were negative on the DB40 cell line. This cell line did not demonstrate the same issue with low event counts. Results from both cell lines agree with other AChR antibody testing and matched expected outcomes (Table 1). Low event counts are denoted in the table by LEC, and unknown values are noted with a U.

<u>Radioimmunoassay Blocking Negative Samples</u>

Of the 102 RIA AChR blocking negative samples, one sample tested positive by the flow methodology on the RD cells. This patient with the flow positive result also tested positive for binding and modulating antibodies and is presumed to have a false negative result by RIA.

All 102 RIA negative patient samples had negative results with the DB40 cell line (Table 2). While the DB40 has a better correlation with the RIA blocking results, this cell line did not yield a positive result for the one sample, patient ID 2 (Table 2), that was positive on the RD cell flow assay and positive by binding and modulating as well.

Table 1. Result table for self-proclaimed healthy donor sera. This table summarizes all available results for binding, blocking, and modulating AChR antibody testing on 122 self-proclaimed healthy donors. Gender and age were available for most patients. All modulating results were obtained by RIA for this group of patients.

ID	Gender	Age	Binding (Pos) ≥ 0.5nM	Modulating RIA (Pos) $\geq 26\%$	Blocking RIA (Pos) $\geq 25\%$	Blocking Flow RD (Pos) $\geq 37\%$	Blocking Flow DB40 (Pos) $\geq 40\%$
1	F	37	0.1	7	6	3	17
2	M	47	0.0	0	5	11	3
3	F	44	0.0	0	7	9	9
4	M	50	0.0	0	8	9	11
5	M	52	0.0	0	6	12	18
6	M	60	0.0	3	4	0	9
7	M	49	0.0	0	5	9	15
8	M	45	0.0	0	4	4	14
9	M	48	0.0	2	4	0	20
10	F	36	0.0	2	0	0	2
11	M	47	0.0	0	8	5	15
12	F	56	0.0	0	6	10	19
13	F	58	0.0	0	2	19	22
14	M	37	0.0	0	2	15	17
15	M	66	0.0	7	4	2	15
16	M	56	0.0	0	2	6	13
17	F	U	0.0	0	8	8	22
18	F	66	0.0	0	5	19	15
19	M	58	0.0	0	2	21	18
20	M	57	0.0	0	2	11	24
21	M	46	0.1	0	0	7	21
22	F	59	0.0	0	9	1	16
23	M	45	0.0	0	5	8	17
24	M	52	0.0	0	4	0	17
25	M	50	0.0	0	8	13	17
26	M	36	0.0	0	5	9	15
27	M	50	0.0	0	3	18	10
28	F	35	0.0	11	7	9	20
29	M	47	0.0	5	3	4	16
30	F	43	0.0	7	3	2	19
31	F	43	0.0	11	2	14	17
32	F	36	0.0	18	2	6	24
33	M	51	0.0	7	4	7	17

Table 1. Continued

ID	Gender	Age	Binding (Pos) ≥ 0.5nM	Modulating RIA (Pos) $\geq 26\%$	Blocking RIA (Pos) $\geq 25\%$	Blocking Flow RD (Pos) $\geq 37\%$	Blocking Flow DB40 (Pos) $\geq 40\%$
34	F	57	0.0	0	0	5	18
35	F	56	0.0	0	0	0	18
36	M	41	0.0	9	0	8	25
37	M	48	0.0	0	0	5	18
38	F	44	0.0	7	0	0	18
39	M	38	0.0	3	0	0	23
40	F	49	0.0	29	0	0	26
41	M	38	0.0	18	0	6	19
42	M	39	0.2	0	0	9	1
43	M	49	0.0	0	0	19	23
44	M	40	0.0	10	0	22	23
45	F	45	0.0	0	0	3	28
46	F	55	0.0	0	10	8	19
47	M	41	0.0	0	13	0	16
48	M	36	0.1	0	7	2	26
49	F	39	0.0	0	5	2	21
50	F	66	0.0	0	5	5	11
51	F	51	0.1	0	1	11	23
52	F	46	0.0	0	8	14	20
53	F	55	0.0	0	1	13	26
54	M	61	0.0	0	6	2	17
55	F	46	0.0	0	5	0	21
56	F	52	0.0	0	0	10	25
57	M	44	0.5	0	2	6	19
58	M	35	0.1	0	1	19	15
59	F	46	0.1	0	0	10	21
60	F	36	0.0	0	0	0	28
61	F	58	0.0	0	0	23	16
62	M	36	0.0	0	0	0	26
63	M	55	0.0	0	0	18	10
64	F	52	0.0	0	0	18	29
65	F	62	0.0	0	0	0	18
66	M	40	0.0	0	0	5	16
67	F	64	0.0	0	0	9	24
68	F	61	0.0	0	14	1	26
69	F	41	0.0	0	9	0	22

Table 1. Continued

ID	Gender	Age	Binding (Pos) ≥ 0.5nM	Modulating RIA (Pos) $\geq 26\%$	Blocking RIA (Pos) $\geq 25\%$	Blocking Flow RD (Pos) $\geq 37\%$	Blocking Flow DB40 (Pos) $\geq 40\%$
70	M	56	0.0	0	8	6	15
71	F	40	0.0	0	15	10	21
72	F	41	0.0	0	14	13	22
73	F	38	0.0	0	3	17	22
74	M	35	0.0	0	8	0	18
75	F	42	0.0	0	3	11	14
76	F	41	0.0	0	0	6	29
77	F	44	0.0	0	3	2	28
78	M	36	0.0	0	4	0	19
79	F	56	0.0	0	0	16	23
80	F	38	0.0	0	0	22	21
81	F	35	0.1	0	5	5	20
82	M	54	0.0	0	4	0	22
83	F	37	0.0	0	0	16	17
84	M	35	0.0	3	0	20	28
85	M	42	0.1	0	0	LEC	0
86	M	38	0.1	3	0	6	23
87	F	39	0.0	2	0	0	22
88	M	40	0.0	0	0	18	19
89	F	39	0.0	3	0	0	20
90	M	66	0.0	0	0	19	17
91	M	45	0.0	0	0	15	0
92	M	42	0.0	0	0	0	3
93	F	57	0.0	0	0	21	6
94	M	34	0.0	0	0	8	11
95	M	51	0.0	0	0	4	4
96	M	39	0.0	0	0	7	22
97	F	38	0.1	0	0	11	22
98	M	53	0.0	0	0	20	24
99	M	45	0.0	0	5	8	0
100	F	45	0.0	0	0	21	5
101	F	44	0.0	0	0	21	17
102	F	45	0.0	0	0	20	22
103	M	44	0.2	0	2	8	9
104	F	44	0.0	0	0	20	3
105	F	37	0.0	0	0	16	9

Table 1. Continued

ID	Gender	Age	Binding (Pos) ≥ 0.5nM	Modulating RIA (Pos) $\geq 26\%$	Blocking RIA (Pos) $\geq 25\%$	Blocking Flow RD (Pos) $\geq 37\%$	Blocking Flow DB40 (Pos) $\geq 40\%$
106	F	35	0.0	0	0	15	28
107	M	47	0.0	0	0	20	4
108	F	42	0.0	0	0	22	9
109	F	46	0.0	0	0	20	19
110	F	38	0.0	0	0	32	25
111	F	56	0.0	0	0	16	17
112	M	43	0.0	0	0	13	8
113	F	49	0.0	0	0	13	18
114	M	55	0.0	0	0	18	19
115	M	47	0.0	0	0	19	22
116	M	U	0.0	0	1	20	14
117	M	39	0.0	0	0	30	26
118	M	40	0.0	0	5	29	26
119	M	45	0.0	0	17	17	5
120	M	47	0.1	0	22	10	13

Table 2. Result table for radioimmunoassay blocking negative patient sera. This table summarizes all available results for binding, blocking, and modulating AChR antibody testing on 102 RIA blocking negative patient samples. Gender and age were available for most patients. Modulating results were obtained by RIA or flow cytometry for this group of patients based upon available methodology on date of testing.

ID	Gender	Age	Binding (Pos) $\geq 0.5nM$	Modulating RIA (Pos) $\geq 26\%$	Blocking RIA (Pos) $\geq 25\%$	Blocking Flow RD (Pos) $\geq 37\%$	Blocking Flow DB40 (Pos) $\geq 40\%$
1	M	73	5.7	37	9	36	24
2	F	96	15.7	30	19	48	29
3	M	63	0.0	0	6	3	7
4	F	90	0.0	0	0	8	6
5	F	55	0.0	0	0	14	6
6	F	63	0.3	6	0	18	3
7	F	55	0.0	7	0	7	0
8	M	64	0.0	0	0	16	0
9	M	68	0.0	0	0	24	0
10	M	8	0.2	0	0	23	0
11	F	67	0.0	0	0	25	1
12	F	73	0.0	0	0	18	0
13	F	24	0.1	0	0	22	0
14	F	33	0.0	6	0	14	0
15	M	78	0.0	6	3	19	1
16	M	32	0.0	3	0	26	0
17	F	81	0.0	0	0	24	1
18	M	62	0.0	4	0	26	0
19	F	72	0.0	2	0	8	0
20	F	63	0.1	1	7	26	0
21	F	72	0.0	2	0	24	0
22	F	47	0.1	2	4	26	0
23	F	47	0.0	0	0	0	0
24	F	28	0.0	0	0	17	0
25	M	62	0.1	16	0	21	4
26	M	70	0.0	0	0	35	0
27	F	7	0.0	0	0	36	0
28	F	75	0.0	0	0	26	6
29	F	59	0.0	0	0	22	0
30	F	20	0.2	0	0	15	7
31	M	53	0.2	0	1	36	1
32	M	68	0.1	0	0	15	3

Table 2. Continued

ID	Gender	Age	Binding (Pos) ≥ 0.5nM	Modulating Flow (Pos) $\geq 46\%$	Blocking RIA (Pos) $\geq 25\%$	Blocking Flow RD (Pos) $\geq 37\%$	Blocking Flow DB40 (Pos) $\geq 40\%$
33	M	52	0.0	0	1	5	7
34	M	81	0.0	9	1	0	6
35	M	72	0.0	0	1	0	3
36	F	66	0.0	17	2	0	13
37	F	17	0.0	5	2	0	9
38	M	84	0.0	0	13	0	4
39	M	72	0.0	0	3	0	7
40	M	60	0.2	2	2	0	3
41	F	65	0.3	4	4	0	4
42	F	81	0.0	11	0	0	0
43	F	83	0.0	0	0	0	8
44	F	50	0.0	3	0	0	4
45	F	26	0.0	0	0	0	0
46	M	61	0.0	0	0	0	5
47	M	64	0.0	0	0	0	3
48	F	76	0.0	0	0	0	5
49	F	61	0.0	0	0	0	8
50	F	64	0.0	0	0	0	0
51	M	35	0.0	0	0	0	4
52	F	58	0.0	8	0	0	16
53	F	49	0.0	0	0	0	4
54	M	65	0.0	0	0	0	11
55	M	66	0.0	0	0	1	7
56	F	48	0.1	2	0	0	7
57	F	78	0.0	0	0	0	9
58	F	22	0.0	7	4	2	6
59	M	45	0.0	1	4	0	7
60	F	56	0.0	0	4	0	7
61	M	65	0.2	0	4	0	19
62	F	29	0.0	0	0	0	12
63	F	41	0.2	8	0	0	20
64	F	66	0.4	0	0	3	9
65	F	58	0.1	0	0	0	12
66	F	57	0.0	0	0	0	8
67	F	47	0.0	8	0	0	10
68	F	56	0.0	13	5	1	20
69	F	61	0.0	6	5	0	16

Table 2. Continued

ID	Gender	Age	Binding (Pos) $\geq 0.5nM$	Modulating Flow (Pos) $\geq 46\%$	Blocking RIA (Pos) $\geq 25\%$	Blocking Flow RD (Pos) $\geq 37\%$	Blocking Flow DB40 (Pos) $\geq 40\%$
70	M	72	0.4	1	5	0	7
71	M	43	0.0	16	5	0	9
72	F	67	0.0	6	5	0	17
73	F	54	0.0	11	5	0	11
74	F	51	0.1	2	6	0	7
75	F	33	0.0	4	6	0	18
76	F	33	0.2	26	6	0	18
77	F	24	0.4	2	6	0	10
78	F	67	0.0	16	6	0	19
79	F	64	0.0	7	6	0	18
80	F	60	0.1	0	2	1	20
81	M	73	0.4	6	2	0	12
82	M	41	0.0	5	2	0	12
83	F	49	0.0	1	0	0	19
84	F	44	0.0	8	0	0	25
85	F	35	0.0	0	0	0	18
86	M	71	0.0	0	0	0	19
87	F	70	0.1	4	0	0	11
88	F	48	0.0	0	0	0	18
89	M	78	0.1	11	0	0	25
90	M	34	0.0	0	0	0	13
91	M	49	0.1	5	0	0	23
92	F	69	0.4	13	0	9	32
93	M	60	0.0	6	4	0	15
94	M	83	0.3	0	0	0	32
95	M	54	0.1	0	0	0	25
96	M	68	13.9	14	5	0	30
97	M	82	0.6	10	0	0	8
98	F	69	1.0	17	0	3	5
99	M	52	2.6	30	0	8	20
100	F	67	1.8	31	5	10	3
101	F	47	0.2	U	0	21	4
102	M	59	0	U	4	12	0

Radioimmunoassay Blocking Positive Samples

Of the 102 RIA positive samples, 9 tested negative on the RD cell flow assay. Two of these nine negative flow AChR blocking sera did not possess detectible levels of binding or modulating antibodies, whereas, the remaining 7 tested positive for binding antibodies and/or modulating antibodies and were likely false negative samples in the flow assay. By establishing an indeterminate interval 4 of these 7 likely false negatives would be reported out as indeterminate. The DB40 cell line had a total of 54 out of the 102 RIA screened positive samples test negative (Table 3).

During the course of this study, the modulating antibody testing methodology in our laboratory changed from RIA to flow cytometry. The reference intervals and assay methodologies are noted in both Table 2 and Table 3.

RD Cell Performance Characteristics

The RD cell flow cytometric blocking assay exhibited a 96.9% overall agreement with the RIA blocking assay. When comparing the flow assay to the RIA assay as a gold standard, this new flow assay has a 91.1% sensitivity and a 99.0% specificity. When comparing the flow assay results to the binding and modulating results in addition to the RIA blocking results, this assay has a slightly higher sensitivity at 93% and a specificity of 100%.

This assay exhibits a positive predictive value of 83.2% and a negative predictive value of 96.8%. Linear regression analysis of RIA versus flow cytometry for the detection of AChR blocking antibodies on the RD cell line confirms a good correlation with an R^2 value of 0.7254 (Figure 4).

Table 3. Result table for radioimmunoassay blocking positive patient sera. This table summarizes all available results for binding, blocking, and modulating AChR antibody testing on 102 RIA blocking positive patient samples. Gender and age were available for most patients. Modulating results were obtained by RIA or flow cytometry for this group of patients.

ID	Gender	Age	Binding (Pos) ≥ 0.5nM	Modulating RIA (Pos) $\geq 26\%$	Blocking RIA (Pos) $\geq 25\%$	Blocking Flow RD (Pos) $\geq 37\%$	Blocking Flow DB40 (Pos) $\geq 40\%$
1	M	83	7.1	43	37	15	13
2	F	65	3.3	34	49	57	15
3	F	74	90.0	32	77	65	38
4	F	11	1.5	37	55	55	41
5	M	55	74.0	32	55	68	52
6	F	75	43.0	37	51	72	66
7	F	46	36.0	46	40	59	29
8	F	78	38.0	61	45	66	59
9	F	48	50.0	51	68	57	47
10	M	80	25.0	62	81	71	66
11	M	73	3.9	38	30	41	0
12	M	58	62.0	58	56	77	66
13	F	47	5.0	51	68	67	36
14	M	66	12.3	40	43	56	7
15	M	57	42.0	36	31	45	55
16	M	61	12.6	47	59	47	8
17	M	78	9.1	59	56	54	13
18	F	44	14.9	54	71	69	27
19	F	88	45.0	29	32	37	37
20	F	43	4.3	45	29	61	40
21	F	90	49.0	61	44	71	47
22	M	72	1710.0	43	51	55	38
23	F	58	3.5	15	50	77	57
24	M	70	10.5	43	59	61	23
25	F	63	103.0	49	37	65	41
26	M	72	14.9	32	62	53	15
27	F	82	21.0	50	61	82	20
28	M	64	56.0	49	60	76	48
29	M	66	13.8	49	77	69	38
30	M	44	42.0	57	72	69	55
31	M	57	84.0	50	68	79	69
32	F	85	79.0	75	89	89	93

Table 3. Continued

ID	Gender	Age	Binding (Pos) $\geq 0.5nM$	Modulating Flow (Pos) $\geq 46\%$	Blocking RIA (Pos) $\geq 25\%$	Blocking Flow RD (Pos) $\geq 37\%$	Blocking Flow DB40 (Pos) $\geq 40\%$
33	F	28	159.0	88	69	54	12
34	F	49	9.9	69	70	63	31
35	M	63	56.0	89	63	57	58
36	F	79	420.0	97	75	54	36
37	F	82	81.0	90	68	68	44
38	F	80	5.6	28	69	14	12
39	M	58	180.0	92	80	68	69
40	M	84	10.2	77	62	43	27
41	F	82	98.0	92	69	75	59
42	F	54	194.0	85	79	46	34
43	F	40	19.0	62	69	38	21
44	F	65	55.0	88	62	62	39
45	M	77	27.0	75	75	57	25
46	M	60	18.0	88	74	58	36
47	F	88	20.0	76	61	50	22
48	F	81	18.0	81	35	46	26
49	M	62	89.0	89	60	68	48
50	M	83	18.0	83	49	48	29
51	M	62	2.5	72	47	55	25
52	F	25	98.0	89	47	50	16
53	F	26	36.0	81	39	69	26
54	M	57	12.2	82	59	50	41
55	M	59	2.2	71	32	56	26
56	F	62	20.0	59	46	37	17
57	M	66	470.0	56	61	5	13
58	M	84	4.3	39	33	20	8
59	M	52	0.2	23	38	23	3
60	F	69	26.0	89	43	48	38
61	M	73	106.0	86	38	69	48
62	F	88	6.3	61	35	38	19
63	M	41	97.0	96	71	67	58
64	F	33	1480.0	93	66	58	27
65	F	74	112.0	88	77	65	61
66	M	75	117.0	95	77	81	69
67	F	50	56.0	93	80	71	45
68	M	68	1.4	53	30	50	8
69	M	76	61.0	80	61	64	36
70	M	68	25.0	72	58	40	29
71	M	53	10.9	72	55	74	77

Table 3. Continued

ID	Gender	Age	Binding (Pos) $\geq 0.5nM$	Modulating Flow (Pos) $\geq 46\%$	Blocking RIA (Pos) $\geq 25\%$	Blocking Flow RD (Pos) $\geq 37\%$	Blocking Flow DB40 (Pos) $\geq 40\%$
72	F	66	16.4	81	61	62	28
73	M	64	66.0	83	56	65	40
74	F	9	2.1	71	57	46	17
75	F	65	8.8	83	43	53	22
76	M	77	14.6	66	52	52	22
77	M	77	25.0	78	47	64	15
78	M	70	42.0	87	47	44	30
79	M	56	24.0	85	47	51	42
80	M	64	10.6	74	26	40	31
81	M	63	17.8	81	28	48	26
82	M	52	0.1	21	27	33	10
83	F	87	27.0	74	48	44	15
84	F	66	9.2	64	44	48	14
85	M	65	21.0	84	59	54	25
86	F	76	6.5	57	42	56	33
87	F	76	15.7	65	38	51	38
88	M	62	54.0	86	39	59	47
89	F	47	12.5	53	39	30	16
90	F	60	25.0	51	42	29	22
91	F	64	85.0	94	69	LEC	41
92	M	62	2.2	34	26	26	12
93	M	58	60.0	88	43	59	24
94	F	76	53.0	81	38	73	55
95	M	70	51.0	91	74	66	42
96	M	49	4.3	55	62	53	26
97	M	56	162.0	91	51	63	52
98	M	33	63.0	91	48	65	39
99	M	75	48.0	85	45	59	44
100	M	78	1.6	63	38	40	14
101	M	85	15.1	66	35	43	22
102	F	40	16.5	78	31	44	24
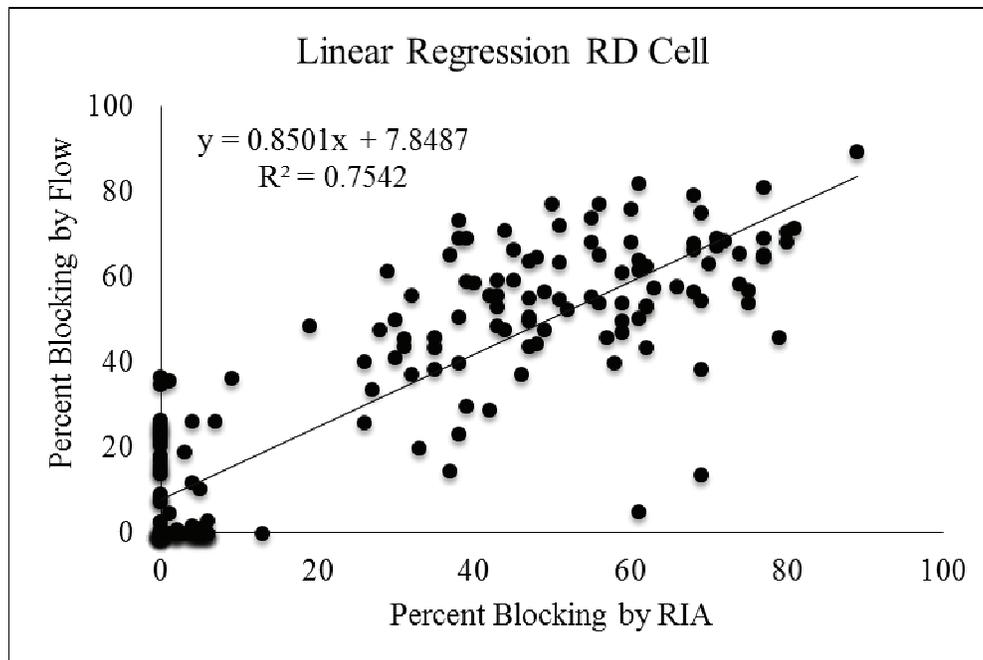
Figure 4. Linear regression RD cell. The linear regression analysis of AChR blocking by RIA versus flow cytometry on the RD cell line indicates good correlation between the two assays.

DB40 Cell Performance Characteristics

The DB40 cell flow cytometric blocking assay exhibited a 73.5% overall agreement with the RIA blocking assay. When comparing the flow assay to the RIA assay as a gold standard, this new flow assay has a 47.1% sensitivity and a 100.0% specificity. When comparing the flow assay results to the binding and modulating results in addition to the RIA blocking results, the sensitivity and specificity calculations as remain the same as stated above. Linear regression analysis of RIA versus flow cytometry for the detection of AChR blocking antibodies on the DB40 cell line confirms a moderate correlation with an R^2 value of 0.5209 (Figure 5).
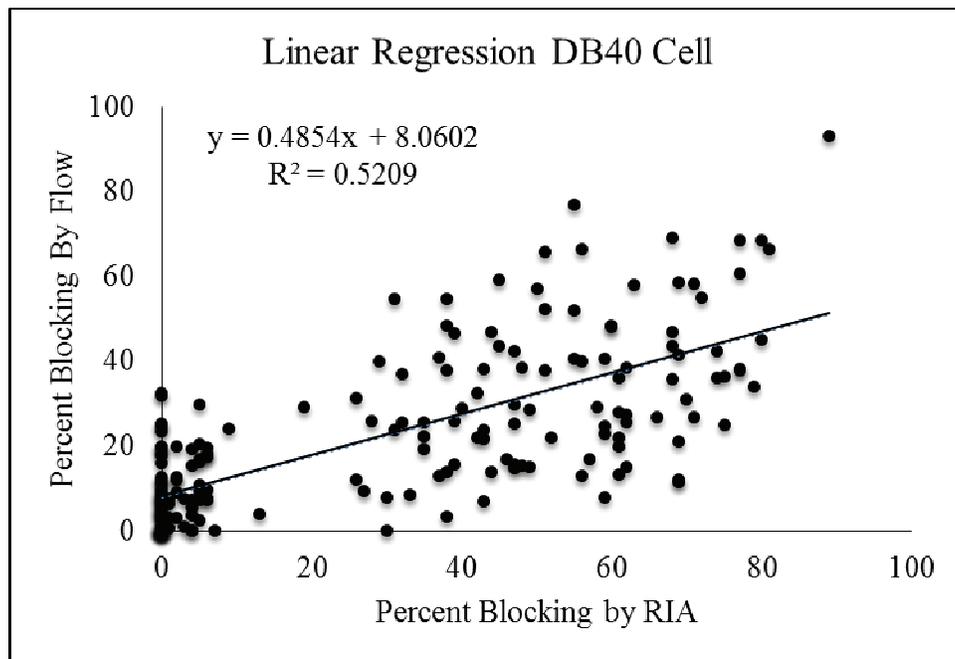
Figure 5. Linear regression DB40 cell. The linear regression analysis of AChR blocking by RIA versus flow cytometry on the DB40 cell line indicates moderate correlation between the two assays.

DISCUSSION

A flow cytometric assay for the detection of AChR blocking antibodies was designed and its performance was evaluated and compared to the performance of the RIA AChR blocking antibody assay. Two cell lines were selected for this study and 324 samples were run using both cell lines once the protocol had been established.

The RD cell line was selected as this cell line is used for the RIA blocking and modulating AChR assays, as well as the flow cytometric modulating assay at ARUP Laboratories. This cell line is readily available and fairly inexpensive. The RD cell, however, does not express the adult AChR subunit ε, it expresses the embryonic γ subunit.

The DB40 cell line is transfected with not only the adult AChR subunit ε, but also the β and δ subunits (26). Although α-Bungarotoxin binds to the α subunit of the AChR, cells with both adult and fetal subunits have different electrophysiological, immuno-logical, and biochemical properties (27, 28, 29). Studies have shown that most MG serum binds well to both the adult and fetal form, or perhaps better to the fetal form which is present in the adult thymus. However, in some cases, patient sera with low AChR antibody titers bind to the adult form with a higher affinity (30). This cell line is proprietary making it expensive and more difficult to obtain.

Evaluation of the performance characteristics of both cell lines on this protocol revealed that the RD cell line compared very well with the current RIA AChR blocking

antibody assay. The DB40 cell line did not perform as well and would not be considered further for the flow assay. A few notable issues were observed with the RD cell line.

Seven samples out of the 324 samples selected for this study are assumed to be falsely negative by flow cytometery. Repeat testing was conducted on all samples with discrepant RIA and flow cytometric results, and were again found to be discrepant. The rational for assuming these samples to be falsely negative is due to positive binding and/or modulating results on those given patient samples.

These samples were all prescreened and selected based on RIA results. This method of sample selection may have resulted in sampling bias that creates a disadvantage for the flow assay when comparing the results to RIA as the gold standard. Theoretically, including samples with positive binding and modulating results, that were negative by blocking RIA, may have resulted in a different sensitivity and specificity calculation. This bias was partially offset by including binding and modulating results in the data analysis.

Access to clinical data for the patients selected for this research would have enhanced this study by confirming which patients were in fact myasthenic. Further studies are being arranged and will include patient histories in order to examine the relationship of clinical presentation and laboratory antibody testing. By testing patient samples with known clinical histories, a better measurement of sensitivity and specificity can be obtained.

To address these discrepant sample results in order to bring this assay in to our lab, we have established an indeterminate reference interval to catch more of those

assumed false negative results. Three levels of reference intervals are now established for this flow assay, positive, negative and indeterminate.

The RD cell line exhibited a phenomenon on two patient samples in which an acceptable cell count of $\geq$ 3000 could not be obtained. Upon repeat testing this issue was again observed. The DB40 cell line did not present this problem. The standard was set at 3000 required events for purposes of consistency and quality for the flow assay, as event counts that consistently fall below this threshold indicate poor cell viability. Further studies may be conducted to determine the significance of these low event counts and evaluate the affected patient results.

Validation of this novel flow cytometric AChR blocking antibody assay for the use in production at ARUP Laboratories required further testing be conducted on this new protocol. Analytical specificity was evaluated by running patient samples that tested positive for voltage-gated calcium channel (VGCC) antibodies, voltage-gated potassium channel (VGKC) antibodies, antinuclear antibodies (ANA), or aquaporin-4 (AQP4) antibodies, on the flow cytometric assay to determine if the interference of these other antibodies would cause false positive AChR blocking antibody results. No interference was noted on any of these antibodies. Interassay and intraassay precision, and carry-over studies were also found to be favorable with no outliers upon data analysis.

The scope of this research was not designed to address a larger issue in the context of MG serological diagnostics. Roughly 15% of generalized myasthenia patients and 50-60% of ocular myasthenia patients are referred to as seronegative as they exhibit no detectable levels of AChR antibodies (31). Muscle-specific receptor tyrosine kinase (MuSK) is also located in the NMJ.

Seronegative myasthenia gravis patients test positive for MuSK antibodies in approximately 70% of cases (32). Double-seronegative patients, those who are negative for both AChR and MuSK antibodies, often test positive for antibodies to the low-density lipoprotein receptor-related protein 4 (LRP4) which has been identified as a receptor that is essential for neuromuscular junction formation (33). Striational antibodies are those that react with epitopes on the muscle proteins titin, ryanodine receptor, and Kv1.4. These antibodies are mainly found in MG patients with thymoma (34).

CONCLUSION

Myasthenia gravis 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. Signaling failure may result from damaged, absent, or blocked AChR. MG can be difficult to diagnose due to the variability of symptoms at disease onset and heterogeneity of patient autoantibody profiles.

The potential for life-threatening complications necessitates the best possible laboratory testing to aid in diagnosis. 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 as AChR antibodies are not observed in a healthy population.

Historically, laboratories have used radio-labeling as the gold standard method for detecting blocking ACHR antibodies as this test has been sensitive and reproducible. Several drawbacks, however, including cost associated with radio-labeling, 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, and its performance was evaluated in comparison to current laboratory standards. The flow

blocking assay was equivalent to the current radioimmunoassay in its ability to detect AChR blocking antibodies. While both assays are comparable, the flow assay offers advantages over radio-assay including enhanced control of QC, and the use of a fluorescent label versus a radio-label 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 viability and cell counts along with measuring fluorescence. These characteristics of the flow cytometric assay allow for improved quality control when compared to the radioimmunoassay.

The 96 well format of the flow assay increases through-put which can potentially decrease the turnaround time of generating patient results. Additionally, the manufacturing and disposal of radioactive iodine is expensive and not environmentally friendly whereas the fluorescent markers utilized in the new assay do not have these issues. The laboratory personnel are also not exposed to radioactive substances when running the flow assay.

In summary, this research describes a new fluorescent flow cytometric AChR blocking antibody assay which is comparable in both sensitivity and specificity when compared to the radioimmunoassay. This assay has a high through-put, is cost effective, and is an environmental and personnel friendly test.

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