DETECTION OF ACETYLCHOLINE RECEPTOR MODULATING ANTIBODIES BY FLOW CYTOMETRY

by

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ABSTRACT

Myasthenia gravis is an autoimmune disease characterized by antibodies against acetylcholine receptors (AChRs) at the neuromuscular junction (NMJ) of skeletal muscle. These antibodies interfere with the transmission of nerve impulses resulting in weakness and paralysis. Due to the variability in symptoms and heterogeneity of autoantibody production in patients with myasthenia gravis, it is essential to make available the best clinical laboratory tests to aid the clinician in diagnosis. One means by which AChR antibodies interfere with nerve impulse transmission is through the effect of antigenic modulation, a process in which antibody-bound AChRs on the postsynaptic muscle cell membrane are internalized and destroyed. The current laboratory assay for the detection of AChR modulating antibodies involves measuring the reduction of expression of radiolabeled AChRs on a human rhabdomyosarcoma (RD) cell line in response to exposure to patient serum.

The goal of this study was to determine the feasibility of detection of AChR modulating antibodies by a new flow-cytometric method rather than the current radioimmunoassay. Two cell lines were investigated: the RD cell line which expresses fetal AChRs and the DB40 cell line which has been transfected with genes for the expression of fetal and adult acetylcholine receptors. Samples tested included sera from 120 self-proclaimed healthy individuals and 100 samples submitted for clinical testing,

50 of which were AChR antibody positive and 50 of which were AChR antibody negative. Results of the flow-cytometric AChR modulating antibody testing on the RD cell line correlated best with results for currently available assays and demonstrated better sensitivity and specificity than the current radioimmunoassay. Results of AChR modulating antibody testing on the DB40 cell line showed slightly decreased sensitivity and specificity, potentially resulting from defects in receptor metabolism due to gene transfection. Detection of AChR modulating antibodies by flow cytometry is feasible and uses an assay format that is more sensitive, specific, and robust with less cost and environmental burden.

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INTRODUCTION

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 (NMJ) in skeletal muscle. These antibodies cause failure of transmission of nerve impulses resulting in progressive muscle weakness.

History

A description of a disorder resembling MG first appeared in 1672 in *De Anima Brutorum* by the anatomist and physician Thomas Willis (1). Willis described subjects who "in the morning are able to walk firmly… or take up any heavy thing, [and] before noon the stock of Spirits being spent… are scarce able to move Hand or Foot." He went on to describe a particular woman who, after speaking at length, would become mute and would not recover the ability to speak for several hours. Willis speculated that the paralysis was due to weakness in a force that was carried in the blood that compelled muscles to move.

Over 200 years passed before the next description of a case likely to have been myasthenia gravis. In 1877 Sir Samuel Wilks, a physician interested in pathology, wrote of the case of a girl (age not stated) who died as a result of bulbar paralysis. Bulbar paralysis is a term applied to dysfunction of muscles innervated by several of the cranial nerves. Wilks performed an autopsy expecting to find necrosis as a result of infarction,

but his findings were significant in that the brainstem appeared healthy. He attributed her death to a case of "hysteria" (1).

Similar cases of fluctuating and recoverable paralysis were described over the next 20 years, and in 1895 Friedrich Jolly described two cases as "myasthenia gravis pseudo-paralytica". From this point on the term "myasthenia gravis" became the name for the disease. He noted that in individuals where one group of muscles was stimulated to exhaustion, other unstimulated muscles in the same individual would exhibit the same weakness. This suggested a circulating factor responsible for the weakness (1).

Several other researchers in the first half of the 20th century continued investigating the disease and found lymphocytic infiltration of the neuromuscular junction, thymic abnormalities, and the discovery by Dr. Mary Broadfoot Walker that symptoms improve with administration of prostigmine (1), a reversible acetylcholinesterase inhibitor (2). These findings culminated in a hypothesis put forth by John Simpson of the National Hospital in London, England that MG was caused by an autoimmune reaction against the neuromuscular junction; thus beginning a more modern understanding of the disease (1).

Clinical Presentation

The major symptom of MG is weakness in specific groups of voluntary muscles. The disease may present clinically in two major ways. Ocular MG is limited to the extraocular and levator palpebrae muscles, while generalized MG 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 (3).

Symptoms of MG most frequently initially manifest in relation to vision. Ptosis (paralytic closing of the eyelid), diplopia (double vision), and blurred vision are most commonly reported. There is no consensus as to whether either ptosis or diplopia usually occurs first, but a slight ptosis may go unnoticed in patients when diplopia is readily apparent. Not all muscles are affected to the same extent, and diplopia may be horizontal, vertical, or diagonal (4).

Bulbar symptoms are seen in muscles innervated by cranial nerves V, VII, IX, X, XI and XII; muscles that control movement of the face, head, and throat. Bulbar symptoms are the second most prevalent relating to the disease. Problems speaking may include a nasal voice or difficulty articulating. Fatigue related to chewing may appear and patients may need to hold their mouths closed by hand at the end of a meal. Difficulty in swallowing or otherwise controlling the mouth may lead to the sensation that food is stuck in the throat, drooling, aspiration of food, regurgitation of liquid through the nose (palatal insufficiency), or ventilatory insufficiency. Weakness of the lips may lead to the inability to whistle or kiss and weakness of the tongue may lead to atrophy with a distinctive longitudinal triple furrowing pattern (4). Weakness of the neck muscles can present as problems in balancing the head, especially if the patient routinely performs work in a bent position. Constant straining of the neck muscles may cause myalgia, normally absent in MG, and may cause the clinician to erroneously search for cervical spine pathology (4).

Weakness in the trunk is almost never the first symptom, but difficulty breathing is often the symptom that causes patients to seek medical attention. Weakness in the legs

often leads to sudden falls, especially on stairs, which brings the possibility of MG to the attention of the clinician. While most patients have both arm and leg symptoms, usually one will predominate and differential weakness can be observed on examination (4).

Half of all patients present with ocular symptoms at the time of diagnosis, with this number increasing to 80% by the end of the first month of illness. Bulbar symptoms are seen in 10% of patients, leg weakness in 10%, generalized weakness in 10%, and 1% of patients present with respiratory failure (5).

Myasthenic crisis is a serious complication of MG characterized by respiratory arrest requiring ventilation and other support measures. Approximately 10-15% of patients will experience this crisis within 3 years of diagnosis (6).

Suspicion of MG does not warrant investigating involvement of the central nervous system. Studies of patients have not shown correlation between symptoms of muscle weakness and decrease in mental function. Also, sera from MG patients containing skeletal muscle acetylcholine receptor (AChR) autoantibodies were not shown to bind AChRs from human brain extract (4).

Pathogenesis

The neuromuscular junction (NMJ) in skeletal muscle resides in a complex environment between the axon terminal of a nerve fiber on the presynaptic side and the highly folded postsynaptic muscle cell membrane. The presynaptic and postsynaptic membranes are separated by a distance of approximately 20 nm, with the intervening space occupied by acetylcholinesterase and other proteoglycans that contribute to synaptic stability (7). The total volume of the NMJ is approximately 50 nm^3, and the

AChRs are clustered at the apices of the folds of the postsynaptic membrane. Estimates on receptor density range from 12,000-24,000 receptors/μm^2 of membrane (7,8). Agrin is secreted by the neuron at the NMJ to stimulate receptor clustering. Agrin is the ligand for the Myotube Associated Specificity Component (MASC)/Muscle Specific Kinase (MuSK) complex. Rapsyn is the protein that links the cytosolic tails of AChRs and is responsible for receptor clustering (8). Rapsyn and AChRs are present in equimolar concentrations in normal muscle cells (7). When an action potential reaches the terminus of the axon, voltage gated calcium channels open in response to membrane depolarization and allow influx of calcium ions into the neuron. The influx of calcium ions causes fusion of ACh vesicles in the neuron with the neuronal membrane. Each vesicle contains approximately 10,000 ACh molecules, and 50-300 vesicles are released with each depolarization. The ACh released into the synaptic cleft quickly diffuses across the gap and binds the AChRs on the postsynaptic membrane apices allowing sodium ions to enter the muscle cell and initiate muscle contraction. Acetylcholinesterase resides in the clefts of the postsynaptic folds and hydrolyzes ACh as it enters the clefts. The clefts act as a sink for the ACh to ensure that the AChRs are stimulated only once by each action potential (8).

The AChRs themselves are pentameric, transmembrane proteins. The 5 receptor subunits are arranged in a rosette around the central ion channel. There are 17 individual receptor subunit types: α1-10, β1-4, γ, δ, and ε. Of the 17 different subunits and all possible combinations, only 2 receptor subunit profiles are expressed in human skeletal muscle AChRs: fetal receptors contain α1, β1, δ, α1, γ subunits and adult receptors contain α1, β1, δ, α1, ε subunits (9). This difference in fetal and adult receptor structure

causes adult AChRs to remain open about 4 milliseconds when stimulated, versus 8 milliseconds in fetal receptors (10). The orders in which the subunits are listed reflect the order of their arrangement when assembled. No two identical receptor subunit types sit adjacent to each other. This will become relevant later in the discussion.

Although the mechanism by which the loss of self-tolerance occurs is not yet understood, much is known about the nature of the autoantibodies, the antigen against which they are directed, and the mechanisms by which these factors disrupt the neuromuscular junction.

The presence of autoantibodies against skeletal muscle AChRs is one of the hallmarks of myasthenia gravis. The most convincing piece of evidence to support this is that patients that undergo plasma exchange to remove these antibodies often experience a dramatic improvement in symptoms (11). These antibodies, depending on epitopic specificity, can bind and crosslink receptor subunits in such a way as to cause the receptors to be internalized and degraded in a process known as receptor modulation. They may also functionally block the binding of the neurotransmitter acetylcholine (ACh), or initiate complement fixation and cause disruption of the postsynaptic cell membrane (12).

Approximately 50% of the antibodies produced in both experimental animals immunized with native $\alpha 1$ subunits and in MG patients are directed against the Main Immunogenic Region (MIR) of the $\alpha 1$ subunit. The MIR is a highly conformational-dependent epitope and immunization with denatured $\alpha 1$ subunits will not elicit the same MIR-oriented immunological response (9). Antibodies directed against the MIR neither allosterically nor competitively prevent the binding of the neurotransmitter with the

receptor. Their pathogenicity stems from their ability to crosslink receptors. Each receptor has two α1 subunits, and thus two MIRs on prominent spurs that jut out from the main body of each subunit domain. This structural feature of the molecule may be responsible for its immunogenicity, subjecting it to detection by potentially autoreactive immune cells (12) (Figure 1).

Each receptor has two MIRs on roughly opposite sides of the molecule, and each MIR autoantibody has two antigen binding sites. Binding of antibodies to adjacent receptors causes the internalization and degradation of the bound receptors with a net loss from the muscle cell surface (receptor loss outpaces receptor expression) (9). Antibodies directed against the MIR are quite pathogenic in that they can cause receptor loss in muscle cell cultures and induce weakness if injected into experimental animals (9).

Blockage of the ACh binding site is an important pathogenic mechanism in myasthenia gravis. Each skeletal muscle AChR has two ACh binding sites, in fetal receptors at the α1:δ and α1:γ subunit junctions and in adult receptors at the α1:δ and α1:ε subunit junctions. Acetylcholine must occupy both sites on a receptor for proper function. Antibody occluding either or both sites is sufficient to inactivate the receptor (9). In Experimental Autoimmune Myasthenia Gravis (EAMG), AChR blocking antibodies cause an acute form of MG lacking NMJ inflammation and necrosis. In patients, these types of antibodies likely explain the "Mary Walker phenomenon" which bears the name of the English neurologist that described it. The nature of this phenomenon is that MG patients treated with cholinesterase blockers and subjected to vigorous exercise quickly have weakness appear in muscles that were not exercised. This effect is consistent with a soluble, reversibly binding agent (such as an antibody) being

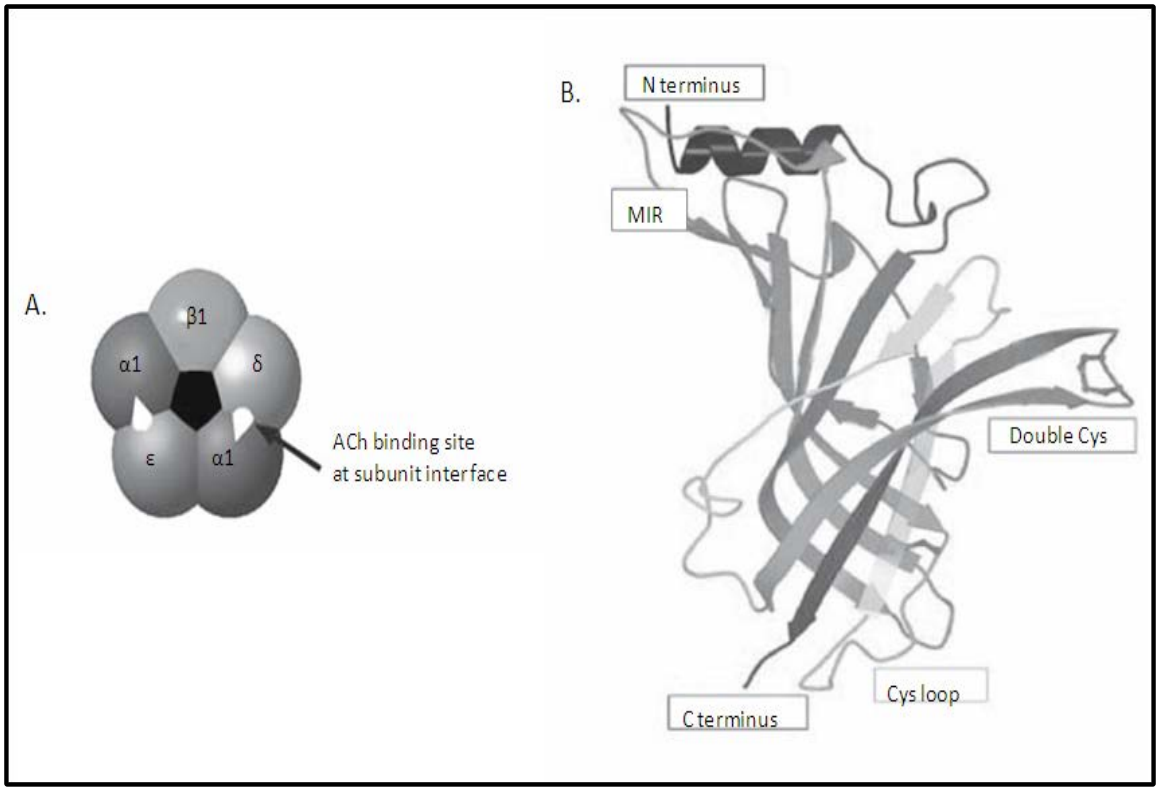

Figure 1. Acetylcholine receptor structure: A) Top view of adult AChR receptor subunit orientation, ion channel (center) and ACh binding sites. B) Diagram of α1 receptor subunit. Springer/Kluwer Academic Publishers. Exploring the Vertebrate Central Cholinergic Nervous System. 2007. Pages 209-235. Neuronal Nicotinic Receptors: History, Structure, and Functional Roles. Jon Lindstrom. With kind permission of Springer Science and Business Media.

displaced from the receptor when its ligand (ACh) is in sufficient concentrations due to presynaptic neuronal release and cholinesterase inhibition (12).

Activation of complement at the NMJ is also an important cause of pathological changes. Inflammation of, and damage to the postsynaptic membrane causes remodeling. This results in not only the loss of furrowing and a decrease in the amount AChRs, but also the loss of voltage gated sodium channels which in turn leads to an increase in the action potential threshold for the muscle cell (11).

In many patients in whom AChR antibodies cannot be detected, Muscle Specific

Kinase (MuSK) is the main autoantigen. In these patients, MuSK antibodies disrupt the agrin/MuSK/rapsyn clustering pathway, leading to ineffective presentation of AChRs and muscle weakness. Mice that lack agrin or MuSK die at birth due to profound muscle weakness and their muscle cells show uniform AChR and rapsyn distribution (7).

The thymus likely plays a role in the pathogenesis of MG, but the mechanisms are not yet fully defined. Most MG patients show thymic histological abnormalities, and of these patients, 70% exhibit thymic lympho-follicular hyperplasia (TFH) that resembles T and B cell germinal centers normally present in only peripheral lymphoid organs. Xenograft of fragments of these abnormal thymi into severe combined immune deficient (SCID) mice results in production of human AChR antibodies in the mice. Thymectomy in MG patients has also been shown to improve the course of disease (12).

Effects of cytokines on pathogenesis of the disorder also appear to be significant. Lymphoid cells isolated and cultured from thymomae of MG patients produce neutralizing antibodies against the cytokines interferon-α and interleukin-12. If thymoma recurs, these antibodies are produced in higher concentration than AChR antibodies suggesting a strong link between anti-cytokine antibodies and thymoma (13). Proinflammatory cytokines such as tumor necrosis factor-α and interferon-γ upregulate expression of AChR, possibly allowing more rapid AChR sensitization once an inflammatory response is initiated (14).

Thymoma is found in 10-15% of MG patients. It is important to distinguish between thymoma (thymic epithelial neoplasm) and thymic carcinoma, which resembles malignancies from other regions of the body. Only thymomas retain the ability to selectively promote intratumorous T-cell development, and only thymomas are associated

with paraneoplastic myasthenia gravis (15). Of patients with thymoma, 10-15% exhibit paraneoplastic syndromes other than MG, and 4-7% of patients with thymoma and MG exhibit more than one paraneoplastic syndrome (5).

As mentioned previously, the mechanism that causes the initial loss of discrimination between self and foreign antigen and initiates autoimmunity is unknown. However, there are several hypotheses that may help explain the problem in the future. One model is that molecular mimicry can induce potentially autoreactive CD4+ T cells to initiate a reaction. Potentially autoreactive CD4+ T cells escape clonal deletion during immune system development and in normal individuals never induce a significant autoimmune response. Microbial antigen fragments can resemble self-antigens and induce potentially autoreactive cells into full autoimmunity (12).

A second model is that a microbial superantigen (SAG) could cause loss of tolerance. In a hyperplastic thymus, there is an abnormal number of dendritic cells (DCs) around which cluster CD4+ T cells. A DC infected with a SAG-producing virus or bacterium could then activate AChR-reactive CD4+ T cells, which would then migrate to the lymph nodes and activate B cells (12).

Lastly, a viral infection of muscle tissue could cause cross-presentation of self-antigen to potentially autoreactive CD4+ T cells that, in the presence of proinflammatory antiviral cytokines, would be induced into full autoimmumnity (12).

Epidemiology

Epidemiological studies of MG have been ongoing since the 1950s, and findings for prevalence of the disease range from 0.5 to 20.4 per 100,000 individuals. No areas of high or low incidence have been identified, no disease clustering can be identified, and the course of disease seems to be rather uniform. Most studies have been on white populations of western European descent (16).

Phillips et al. (16) performed a study in Virginia in the mid-1980s and found MG prevalence around 14 per 100,000, with a female to male ratio of 1.5:1, and median onset age for women of 41.7 years and 60.3 years for men. This study confirms the truism that MG generally affects women earlier in life than men (14).

Prevalence of MG has also been steadily increasing for the last several decades, most likely due to a combination of improved surveillance, awareness of the disease and improved treatments allowing patients to live longer (14).

Annual incidence of MG is 3-5 per million with a higher prevalence seen in urban areas versus rural (4).

Seronegative Myasthenia Gravis (SNMG) is MG where there are no detectable levels of AChR antibodies. Roughly 15% of generalized MG patients fall into the SNMG category, while 50-60% of ocular MG patients are seronegative. Hoch et al. reported in 2000 that some SNMG patients had MuSK antibodies, and it has since been found that 30-70% of SNMG patients are positive for MuSK antibodies (14).

In 2009, Alshekhlee et al. published a study using a nationwide database of inpatient disease classification codes to identify patients with diagnosis of myasthenic crisis (17). Of 5,502 patients, the authors found that the incidence in women was two to

three times higher than men in the first 5 decades of life. Men, however, had a higher incidence than women in the 6th, 7th, and 8th decades of life. The overall mortality rate for MG while hospitalized was 2.2%, with a mortality rate of 4.47% for those patients experiencing myasthenic crisis (17).

Clinical Diagnosis and Laboratory Testing

Clinical diagnosis of MG is carried out by first recording an accurate patient history, taking note of periods of fluctuating weakness. The historical data is then corroborated with data from physical exams and laboratory studies.

Pharmacological testing can assist in diagnosing myasthenia gravis. Edrophonium chloride (Tensilon) is an acetylcholinesterase inhibitor with onset of action of about 30 seconds and duration of about 5 minutes. Edrophonium temporarily increases the amount of ACh at the NMJ, and allows the clinician to observe recovery of function in myasthenic patients. This test is most useful in patients with symptoms of ocular MG where muscle performance may be measured objectively (3). Care must be taken when administering edrophonium, as there is a risk for bronchospasm, bradychardia, and extreme gastrointestinal side effects. Sensitivity for edrophonium testing is about 90%, specificity is not easy to determine due to improved function after administration in other neuromuscular diseases. Neostigmine is another acetycholinesterase inhibitor of longer duration that is an alternative for testing in children (5).

Electrophysiological testing directly stimulates the nerve side of the NMJ and measures the response of the muscle. One such study is termed repetitive nerve stimulation (RNS). In such a study, a motor nerve is stimulated at a low rate of 2-5 Hz,

which depletes the stores of ACh at the neuromuscular junction. This reduces the likelihood of an action potential reaching the muscle, and causes failure of some of the transmissions. These decremental responses are compared to baseline values, and the test is considered positive if there is a decrease of more than 10% between the baseline and 4^{th} stimulation (3).

Care must be taken in RNS that no acetylcholinesterase inhibitors be given for at least 12 hours prior to the RNS test; that proximal muscles such as the facial, trapezius, or deltoid muscles be avoided as they are more likely to give erroneous results; and that careful attention to proper technique be paid. If performed properly, RNS testing only shows a sensitivity of 60% for myasthenia gravis (3).

Single fiber electromyography (SFEMG) is a technique in which multiple muscle fiber responses are measured from stimulation of a single nerve. The difference in time to contract between individual fibers is called the neuromuscular jitter. This technique should always be performed in a clinically weak muscle. Usually testing is begun in the extensor digitorum communis muscle, if findings there are normal then a facial muscle should be studied next. When limb and facial muscles are studied in conjunction, SFEMG shows 97% sensitivity for myasthenia gravis. Other neuromuscular disorders will produce abnormal transmission results, and SFEMG must be performed by a qualified expert using specialized equipment to ensure result validity (3).

Serological assays for AChR binding, blocking and modulating antibodies exist. One of the most useful in clinical practice is the AChR binding antibody assay. This is a radioimmunoprecipitation assay that uses soluble AChR labeled with radio labeled ^{125}I α-bungarotoxin (BGTX). Patients with early-onset MG with generalized weakness tend to

have the highest responses with this assay format (18).

The soluble receptor used in the AChR binding assay and the heterogeneous nature of patients' antibody profiles can make detection of antibodies in patients vary. One source of receptor for this assay is the TE671 cell line, a human rhabdomyosarcoma (RD) cell that expresses fetal acetylcholine receptor (20); another source is human cadaver muscle. The effect of soluble receptor source was studied at ARUP Laboratories in SLC, Utah. The study included three receptor sources: human muscle extract, RD cell extract, and an 80/20% mixture of RD/muscle extract. Findings were that for 96% of patients, there was no difference in result. For 4% of patients, however, result interpretation changed depending on pure receptor source and the highest sensitivity was attained with the mixture (20).

Acetylcholine receptor blocking antibodies can be measured by their ability to inhibit binding of BGTX in culture. There is some debate as to whether these antibodies competitively block the neurotransmitter binding site, or if they allosterically inhibit the toxin binding (18).

Acetylcholine receptor modulating antibodies can be measured by exposing BGTX labeled RD cells in culture to sera from MG patients. Radiolabeled receptors will be internalized and degraded, and the ratio of radioactivity in the culture supernatant versus the cell pellet can be used to determine percent receptor modulation (21).

Recent research has focused on flow cytometric methods for determining the presence of AChR modulating antibodies, as well as possibly binding and blocking antibodies. These methods use fluorescent labels rather than radiolabels, thereby eliminating a regulatory and environmental burden (19).

A meta-analysis of literature surveying efficacy of MG testing found that edrophonium testing was reported to be 92% sensitive for ocular MG and 88% sensitive for generalized MG, and 97% specific for both forms of disease. The variability in the selection of nerve-muscle pairs tested in RNS studies made it impossible to draw conclusions regarding testing outcomes for this method. However, generally the data suggested that RNS is a poor diagnostic tool for ocular MG and outcomes being better for generalized MG if multiple muscle-nerve pairs are tested. Although there was some question as to whether the methodology was practiced effectively in all studies, SFEMG sensitivity and specificity were high for ocular MG but data were limited for generalized MG, yet did demonstrate high specificity. For antibody studies, the authors did not differentiate AChR binding, blocking, or modulating antibodies. Antibody testing showed 96% sensitivity for generalized MG, and 44% sensitivity for ocular MG. Very good specificity of 98-99% was reported for antibody testing in any form of myasthenia gravis (22).

Treatment and Outcomes

Without treatment, MG progresses in most patients and 66% of those initially presenting with ocular symptoms will progress to generalized myasthenia gravis. After 2 years, 14% will remain with strictly ocular symptoms. Those left untreated will experience progressive weakness involving more muscle groups with some developing fixed weakness with muscle atrophy, while 25-31% will succumb to the disease (23). Advances in disease treatment and maintenance over the past few decades, however, have essentially reduced mortality to zero (4).

The first tier treatment for MG involves cholinesterase inhibitors. These drugs bind acetylcholinesterase reversibly, and have minimal central nervous system side effects since they do not readily cross the blood-brain barrier (5). Pyridostigmine is one of the most frequently used inhibitor drugs as both initial therapy for new patients, and long-term therapy for patients with mild symptoms. Adverse effects include GI tract hypermotility, increased sweating, excessive mucous membrane secretions, and muscle cramps. Caution should be exercised in administering cholinesterase inhibitors to MuSK antibody positive patients as they frequently have ACh hypersensitivity (24).

Plasma exchange involves removing antibodies from patient plasma via membrane filtration or centrifugation. It can be used to alleviate symptoms on a short-term basis in patients experiencing myasthenic crisis, to induce remission of symptoms prior to surgery, or to alleviate symptoms during the initiation of immunosuppressive therapy (24).

Intravenous immunoglobulin (IvIg) therapy is recommended for similar circumstances as that of plasma exchange, i.e., short term relief of serious symptoms. No long-term benefit has been proven for IvIg in MG cases that are mild to moderate (24).

Thymectomy is of little benefit to patients with mild MG, but patients with more severe MG are 3.7 times more likely to undergo remission with surgery than without. Thymectomy is always indicated in MG patients with thymoma, and prognosis is dependent on early and complete removal of the tumor (24).

Immunosuppressive drugs are used in patients that do not respond well to other therapies. The side effects of various immunosuppressants need to be monitored and taken into account before escalating therapy if the patient does not respond (24).

Summary

Due to the variability of initial symptoms, heterogeneity of patient autoantibody profiles, potential for grave complications as the disease progresses, and the need for the best available clinical laboratory results to aid in disease management, I investigated the possibility of adapting a new flow cytometric method for the detection of AChR modulating antibodies in the clinical laboratory.

MATERIALS AND METHODS

Suspension-Stained Cells

Sera from 97 self-proclaimed normal healthy individuals and 118 samples submitted for AChR modulating antibody testing were de-identified and tested in accordance with University of Utah Institutional Review Board guidelines. Rat anti-human AChR monoclonal antibody 210 (mAb210) was purchased from Abcam (Cambridge, MA) and goat anti-rat IgG Alexa-488® secondary antibody was purchased from Invitrogen (Carlsbad, CA). Human rhabdomyosarcoma (RD) cells (CCL-136; ATCC. Manassas, VA) expressing fetal AChRs were cultured in 175 cm^2 flasks at 37°C, 5% CO_2, in a 95% humidity incubator to confluence. Culture medium was purchased from ARUP Laboratories Reagent Lab (Salt Lake City, UT) and consisted of Eagle's Minimal Essential Medium (EMEM) 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 1x phosphate buffered saline (PBS) containing 9mM ethylenediaminetetraacetic acid (EDTA), centrifuged at 500g for 5 minutes at room temperature, decanted, resuspended in culture medium, counted, and normalized to a concentration of $1x10^6$ cells per milliliter. Harvested cells were dispensed into 96 well tissue culture plates at a concentration of $1x10^5$ cells per well. Cells to serve as background secondary antibody or maximum receptor staining

standards were exposed to 4 µL cell culture medium (no serum exposure). Assay control and test patient cells were exposed to 4 µL of the appropriate sera. The plate was then incubated overnight at 37°C, 5% CO_2, in a 95% humidity incubator. The following morning the plate was decanted, cells were dissociated with cold PBS-EDTA, centrifuged at 500g for 5 minutes and washed with cold assay buffer (2% bovine serum albumin in HBSS without Ca^{2+} or Mg^{2+}). All cells were then labeled for 30 minutes on ice with 2 µg/mL mAb210 in assay buffer, except background staining cells which received assay buffer alone. Cells were then washed twice and labeled with 4 µg/mL Alexa-488® conjugated goat anti-rat IgG, including background staining cells. Cells were again washed twice, resuspended in assay buffer, and analyzed on a BD FACSCanto II cytometer using Diva software. Cell populations were gated to normalize fluorescence against nonspecifically stained cells and to exclude cell fragments and aggregates. Acetylcholine receptor percent modulation was calculated by comparing the proportion of gated cells that fell above the fluorescence cut-off to the maximally stained assay standard using the following equation:

$$(1-((\text{Sample} - \text{Background})/(\text{Maximum} - \text{Background})))*100\% \qquad \text{[Equation1]}$$

Assay reference range was established by Receiver-Operator Characteristic (ROC) Curve analysis of flow cytometric versus 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, Inc. Cary, NC). Results of the new assay were then compared to results of the current AChR modulating antibody

assay and other related AChR antibody assays for assessment of performance.

Adherent-Stained Cells

Sera from 120 self-proclaimed normal healthy individuals and 100 samples submitted for AChR modulating antibody testing were de-identified and tested in accordance with University of Utah Institutional Review Board guidelines. Samples were assayed on both the fetal AChR-expressing RD cell line and the DB40 (Isis Innovation, Ltd. Oxford, UK) cell line for the presence of AChR modulating antibodies. The DB40 cell line is derived from the same rhabdomyosarcoma cell line as the RD cell, but has been transfected with genes for the AChR epsilon subunit, and thus expresses both fetal and adult acetylcholine receptors (19). Both cell lines were separately cultured and plated as previously described, with the addition of 0.5 mg/mL geneticin (Gibco, Grand Island, NY) to the DB40 cell culture medium. The AChR modulating antibody assay set-up and overnight incubation were retained for the adherent cell protocol, but assay procedure following the overnight incubation was modified to improve sensitivity, efficiency, and cell viability. 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 HBSS without Ca^{2+} or Mg^{2+}) were added to background stain wells, followed by addition of 100 µL of 2 µg/mL mAb210 in assay buffer to all remaining control and patient wells. Cells were incubated for 30 minutes in 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 4 µg/mL APC-conjugated goat anti-rat IgG (BD Biosciences,

San Jose, CA) for 30 minutes in dark at room temperature. Cells were again washed, and 100 µL 9 mM EDTA-PBS was added and allowed to incubate 10 minutes in 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 phosphate buffered saline. The fixed cell suspension was then transferred to cytometer tubes and analyzed on a BD FACSCanto II cytometer using Diva software.

Results for RD and DB40 cell AChR modulation were calculated according to three different schemes: 1) In Scheme A, cursor gates were set to normalize populations against background fluorescence. The proportion of gated cells above background was taken as a measure of AChR expression. Cell populations in control and test samples were then compared to a maximally stained assay standard. 2) Scheme B used the same gate settings and calculations as in Scheme A, but compared mean fluorescence of standard, control, and test sample cell populations rather than population distribution. 3) Scheme C compared fluorescence of all gated events in standard, control and test samples regardless of background fluorescence. Result calculation for all three schemes used the equation previously described. Reference ranges for all three calculation schemes on both cell lines were generated as previously mentioned and results were compared to the current clinical assay for assessment of performance.

Serial two-fold dilutions of an AChR modulating antibody positive specimen were made in cell culture medium and tested for linearity and possible quantification. Results were calculated according to the scheme that provided optimal correlation with the current assay.

Analytical specificity was assessed by testing 21 samples for AChR modulating

antibodies that also tested positive for anti-nuclear antibodies (ANA), voltage-gated calcium channel (VGCC) antibodies, or voltage-gated potassium channel (VGKC) antibodies.

RESULTS

Suspension-Stained Cells

Results of the ROC curve analysis suggested a positive cut-off of 54% modulation or greater (Figure 2). This cut-off was increased to 60% to eliminate one false positive in the normal sample group (55% modulation). This adjustment did not yield any false negative results in the AChR modulating antibody positive samples. Result distribution is illustrated in Figure 3. Result correlation between the suspension-stained flow-cytometric assay and the current radioimmunoassay is illustrated in Figure 4. The ROC curve in Figure 2 shows an area under curve (AUC) value of 0.84, indicating good sensitivity and specificity at the 54% modulation cut-off.

The result distribution illustrated in Figure 3 was calculated with the 60% modulation cut-off. The figure shows good separation between results of the normal donor samples and the majority of the AChR modulating antibody positive samples. However, the results from the clinical negative samples show a surprising number that test positive by the new assay. One sample of the normal donors did test positive (62% AChR modulation). It was later determined that this individual had been diagnosed with myasthenia gravis in the past, but the individual felt normal at the time of sample collection and symptoms were in remission.

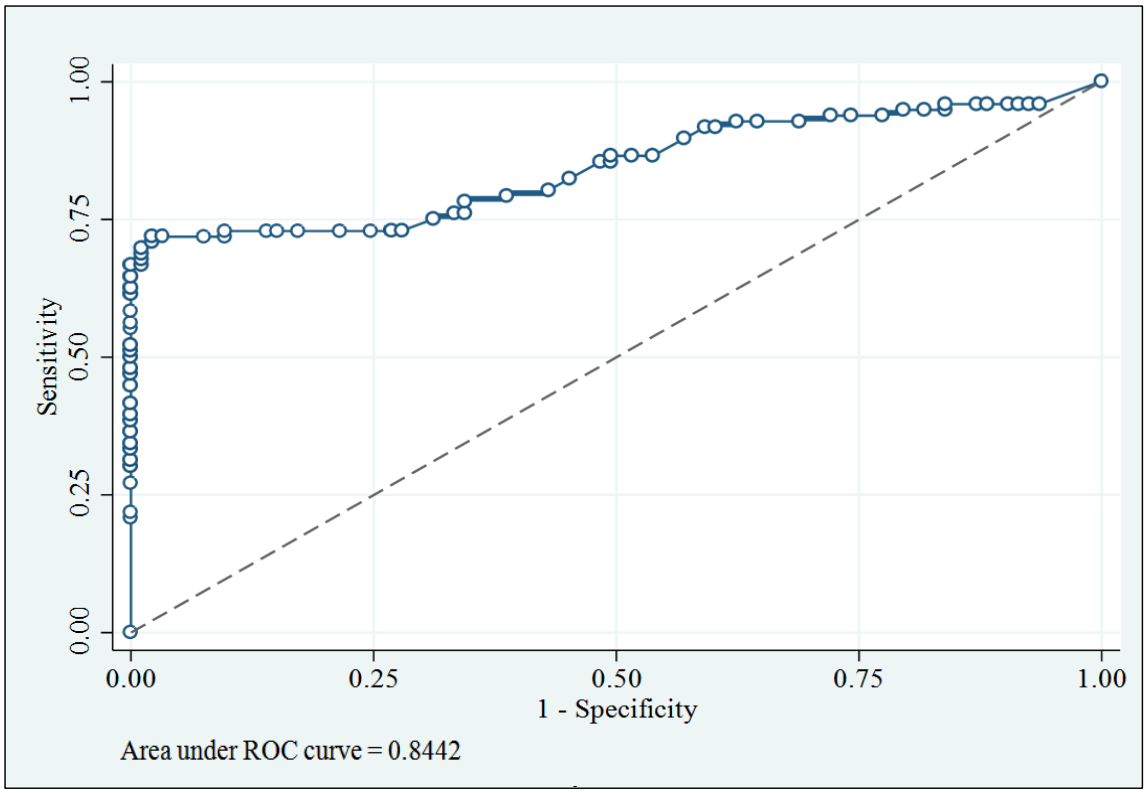

Figure 2. RD Cell Suspension-stained Receiver Operator Characteristic (ROC) analysis. AChR modulating antibody RD cell suspension stain results versus results from the current assay

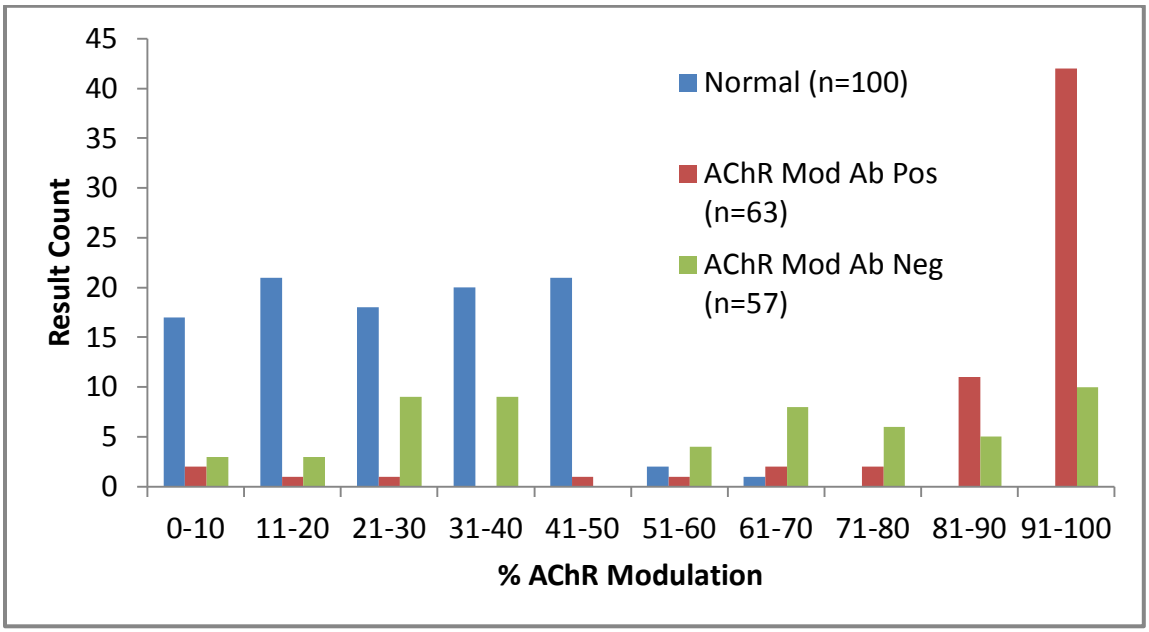

Figure 3. RD Cell suspension-stained result distribution. Result distribution for the AChR modulating antibody assay by flow cytometry.

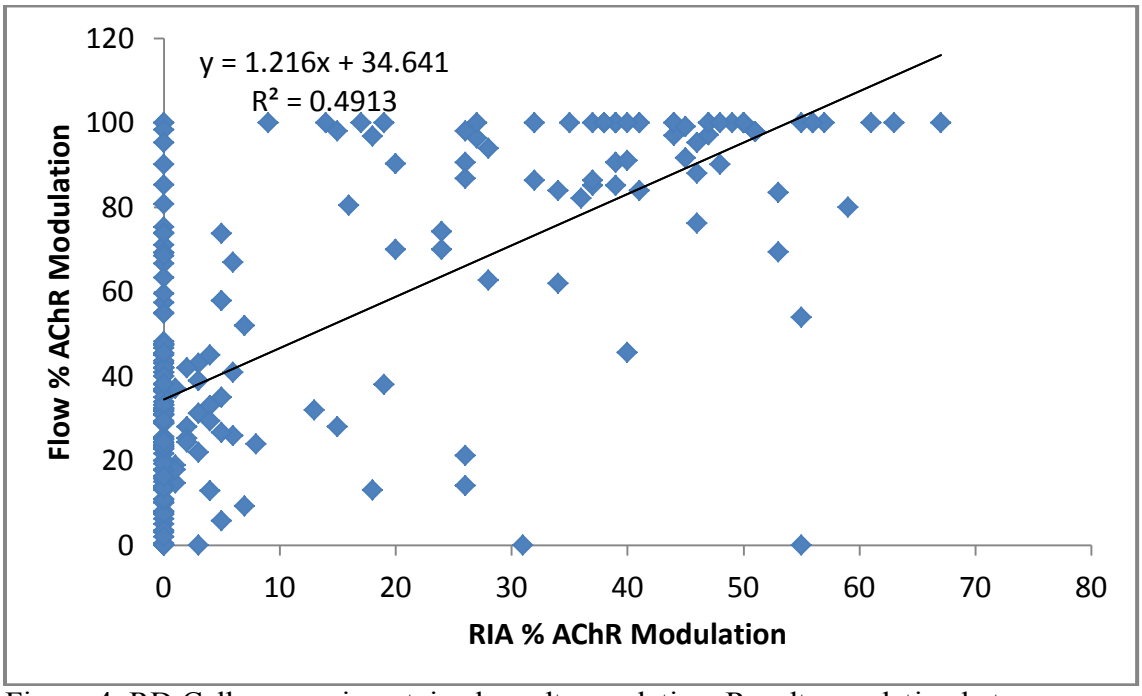

Figure 4. RD Cell suspension-stained result correlation. Result correlation between AChR modulating antibody suspension stain flow cytometric assay and the current radioimmunoassay.

The linear regression plot in Figure 4 demonstrates poor result correlation (R^2= 0.49) between the new assay and the current assay due to the use of different reference ranges between the two assays and the disagreement in a number of qualitative results.

Results for the flow cytometric assay were then compared qualitatively to those of the current AChR modulating and soluble AChR binding antibody assays. When compared to the current modulating antibody assay, the new assay demonstrated 83% agreement, 90% sensitivity, and 81% specificity. Compared to the soluble AChR binding assay, the new assay demonstrated 77% agreement, 65% sensitivity, and 93% specificity. The current AChR modulating antibody radioimmunoassay shows 64% agreement, 43% sensitivity, and 89% specificity against the soluble AChR binding assay. These findings are summarized in Table 1.

Table 1: Comparison of performance of AChR modulating antibody suspension-stain by flow cytometry with current AChR antibody radioimmunoassays

	Agreement	Sensitivity	Specificity
AChR modulating antibody suspension-stain vs. AChR modulating antibody RIA	84%	91%	81%
AChR modulating antibody suspension-stain vs. AChR binding antibody	78%	66%	93%
AChR modulating antibody RIA vs. AChR binding antibody	65%	45%	90%

Results of the two AChR modulating antibody assays were compared to the AChR binding assay due to the wide acceptance of the binding assay as the most sensitive clinical laboratory test (25). Overall, the flow cytometric assay shows better correlation with the AChR binding antibody assay than the current assay, but the actual values appear lower than desirable due to inclusion of samples with extraordinary result profiles. Of the 118 clinical samples included in this part of the study, 65 were samples that were selected due to discrepant AChR antibody results (positive binding, negative modulating, or vice versa). Within the group of clinical samples, 33 displayed discrepant qualitative results between either the new flow cytometric assay, the current radioimmunoassay, or the soluble AChR antibody assay. However, 26 of these 33 discrepant samples were negative by the current assay and positive by the new flow cytometric assay with one or more corroborating positive results from other AChR antibody assays.

Adherent-Stained Cells

Based on the experiences with the suspension-stained cells, the following was observed: 1) the cell line used is adherent, and when dissociated is apparently readily subject to anoikis; 2) the suspension staining was initially conducted on ice in an attempt to minimize AChR loss due to turnover, but colder temperatures were observed to have a profoundly negative effect on cellular integrity; 3) there was a sub-optimal separation of receptor-associated fluorescence between background and maximally stained cell populations; and 4) any time interval between finishing cellular preparation and instrument analysis had a deleterious effect on cellular integrity.

In response to these observations the procedure was changed to be performed at room temperature with cells still attached in a culture plate, a brighter fluorophore-labeled secondary antibody was used, and cells were paraformaldehyde-fixed immediately following staining and dissociation steps. Effects of these modifications can be seen in Figure 5.

The effects of the assay modification illustrated in Figure 5 show a much larger proportion of cells that stain above background fluorescence (population to the right of the cursor gate). The proportion of cells staining above background prior to assay modification was approximately 10% of gated events and increased to approximately 30% after modification. This is attributed mostly to improved cellular integrity due to being kept closer to physiological temperature and remaining attached to a solid substrate during staining. This increase in cell staining most likely contributes to the robustness, sensitivity, and reproducibility of the assay.

Results comparison of the current assay results versus results of the three

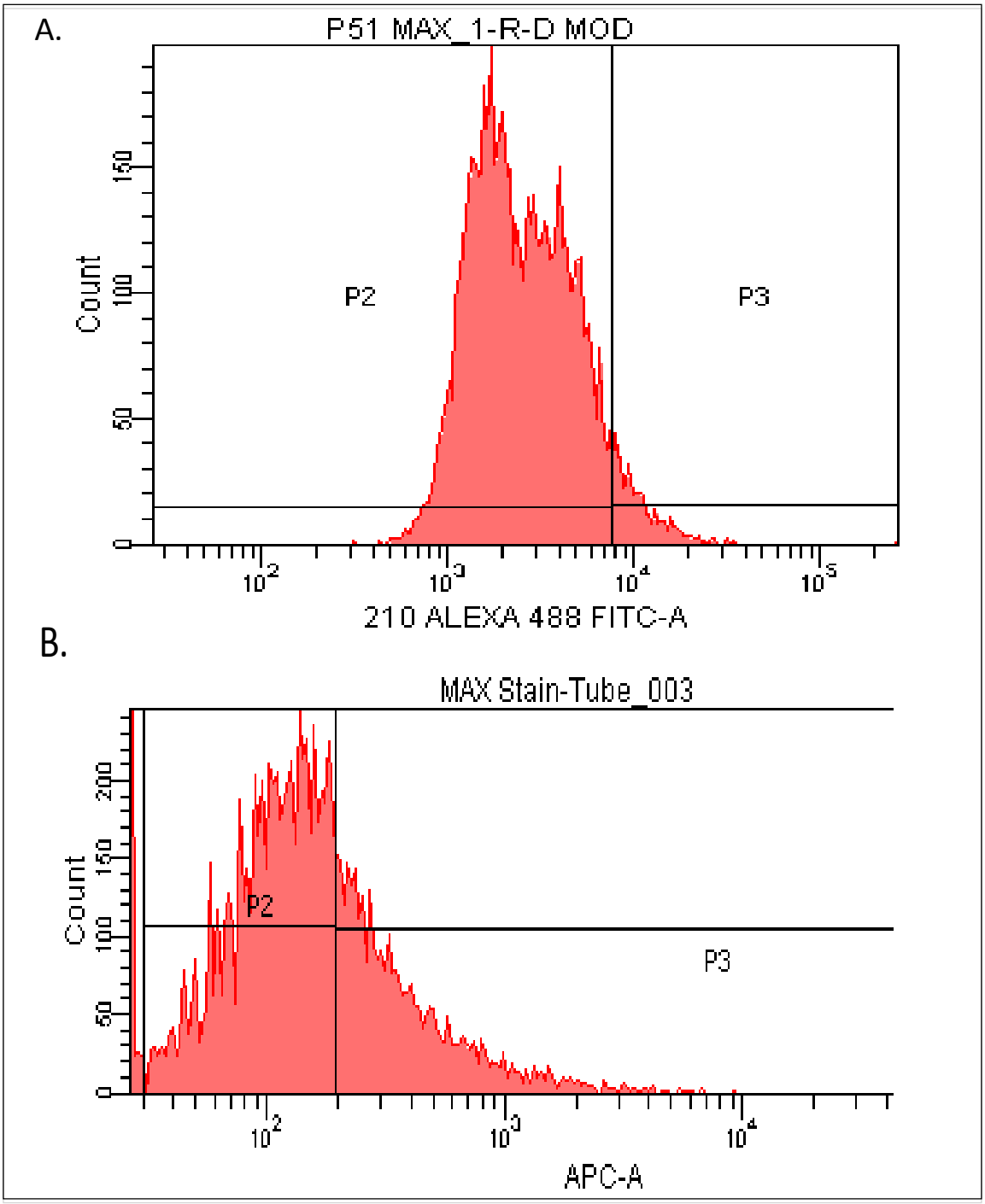

Figure 5. Effects of assay procedure modification. A) Maximally stained assay standard before procedure modification and B) after.

calculation schemes for the two cell lines can be found in Table 2.

The values in Table 2 show that any one of the three calculation strategies combined with either of the two cell lines provides results that compare favorably with the current assay. However, the RD cell line when combined with result Calculation Scheme B provided the closest result match to the current assay. For the DB40 cell line, Calculation Scheme B also provided the most favorable result comparison with the current assay.

Results of adherent RD cell ROC curve analysis are illustrated in Figure 6. Result distribution for RD cell adherent stain Calculation Scheme B is illustrated in Figure 7. Result correlation of adherent RD cell adherent stain Calculation Scheme B with those of the current assay is illustrated in Figure 8.

The ROC curve plot in Figure 6 shows an AUC value of 1.00, indicating very good sensitivity and specificity for adherent RD cell AChR modulating antibody results.

Figure 7 illustrates AChR modulating antibody result distribution for the RD cell line calculated using mean population fluorescence after background staining normalization (Scheme B). Positive cut-off for this calculation scheme was determined by ROC analysis to be 46% modulation or greater. This figure illustrates the stark separation of results for negative and positive samples. The majority of the normal and AChR modulating antibody negative sample results display little to no receptor modulation while the majority of the positive samples display near-complete 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 8 shows good result correlation (R^2= 0.84)

Table 2. Qualitative performance evaluation results for the AChR modulating antibody assay on adherent RD and DB40 cell lines versus the current assay.

RD Cell	Agreement	Sensitivity	Specificity
Calculation Scheme A	97%	90%	99%
Calculation Scheme B	99%	98%	99%
Calculation Scheme C	95%	90%	96%
DB40 Cell			
Calculation Scheme A	91%	82%	94%
Calculation Scheme B	97%	94%	98%
Calculation Scheme C	95%	88%	98%

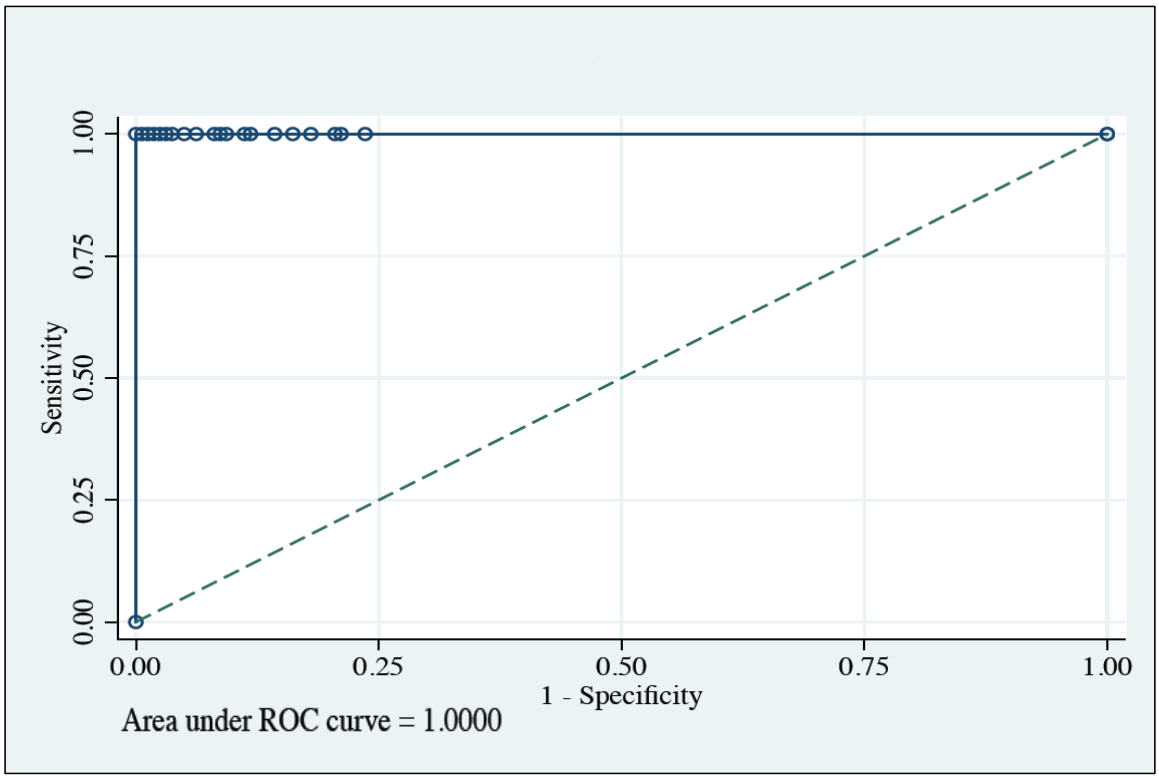

Figure 6. RD cell adherent-stained ROC analysis. Receiver Operator Characteristic curve for AChR modulating antibody RD cell adherent stain results versus results from the current assay. Positive cut-off at 46% modulation.

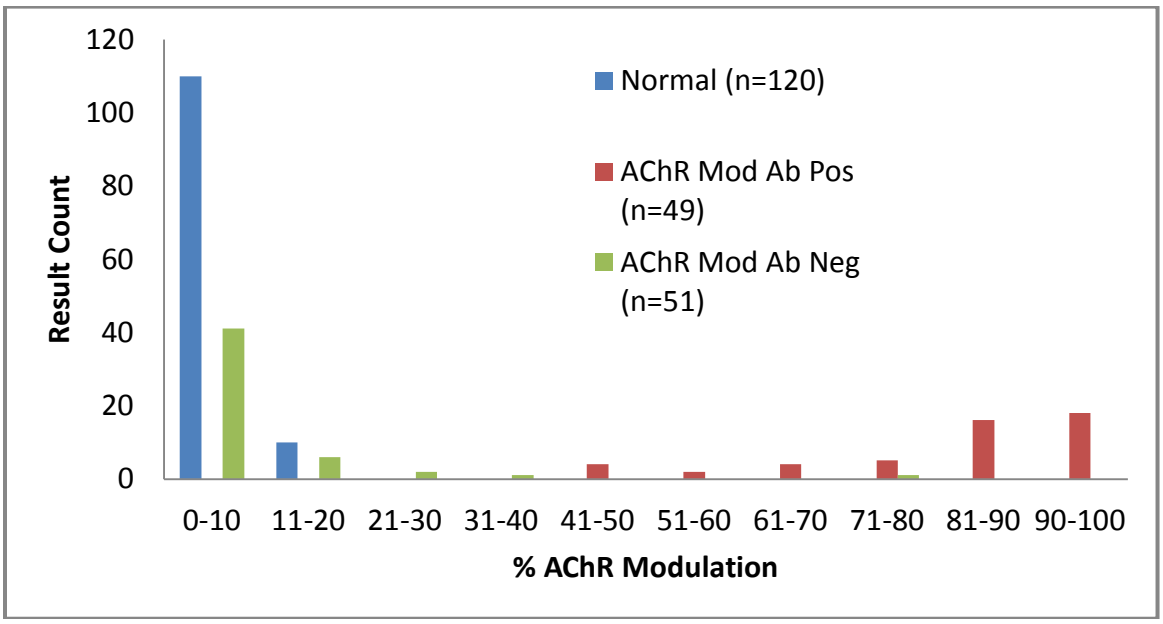

Figure 7. RD cell adherent-stained result distribution. Result distribution for adherent RD cells calculated using mean fluorescence of gated cells after background fluorescence normalization (Scheme B).

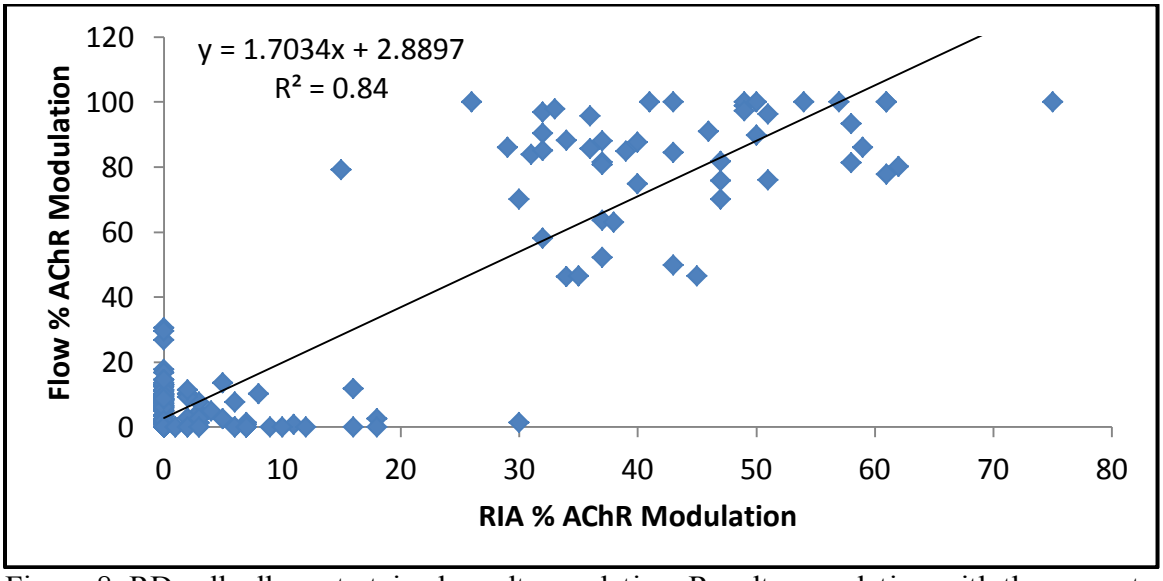

Figure 8. RD cell adherent-stained result correlation. Results correlation with the current assay. Results calculated using mean fluorescence of gated cells after background fluorescence normalization (Scheme B).

between results for RD cell Calculation Scheme B and those of the current assay.

Results of adherent DB40 cell ROC curve analysis are illustrated in Figure 9. Result distribution for DB40 Cell Calculation Scheme B is illustrated in Figure 10. Result correlation of adherent DB40 Cell Calculation Scheme B with those of the current assay is illustrated in Figure 11.

The ROC curve plot in Figure 9 shows an AUC value of 0.97, indicating very good sensitivity and specificity for adherent DB40 cell AChR modulating antibody

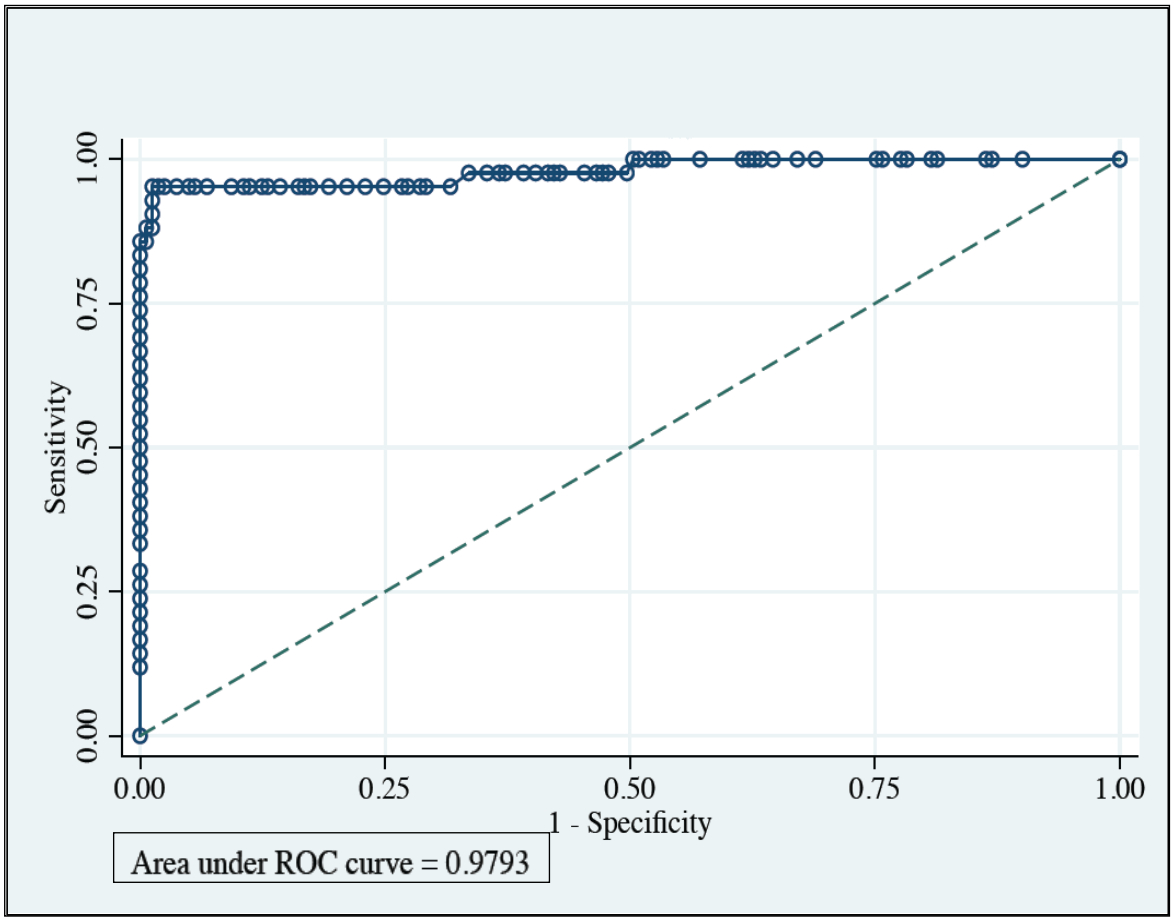

Figure 9. DB40 cell adherent-stained ROC analysis. ROC curve for AChR modulating antibody DB40 cell adherent stain results versus results from the current assay. Positive cut-off at 25% modulation.

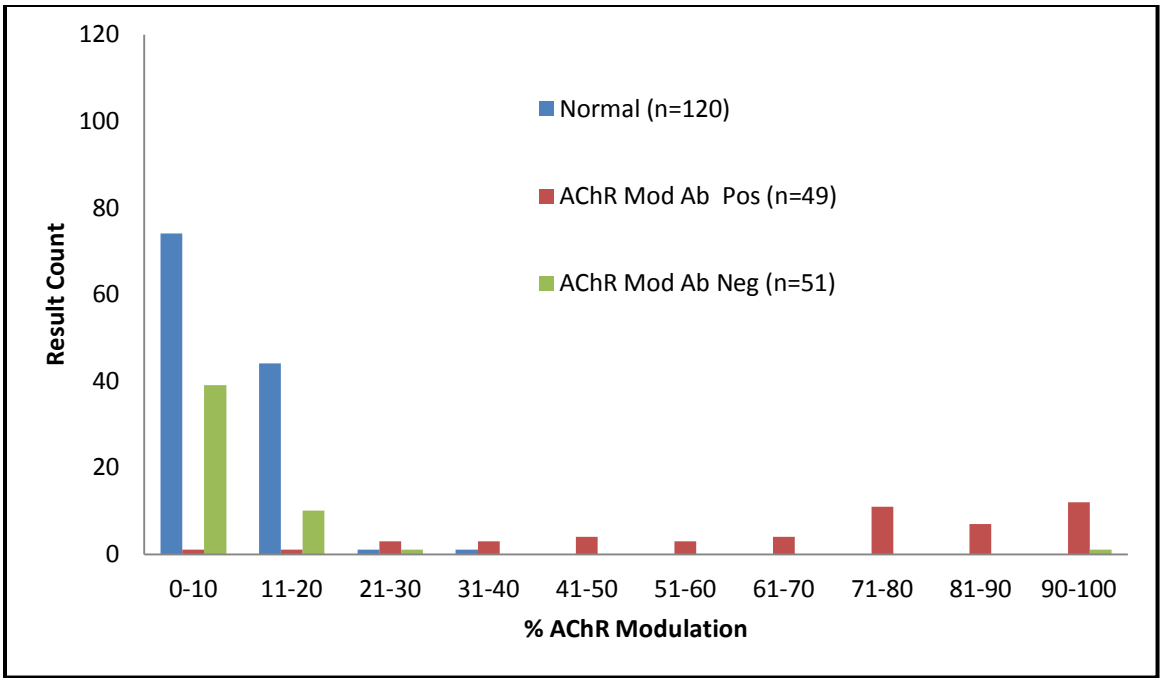

Figure 10. Flow cytometric result distribution for adherent DB40 cells calculated using mean fluorescence of gated cells after background fluorescence normalization (Scheme B).

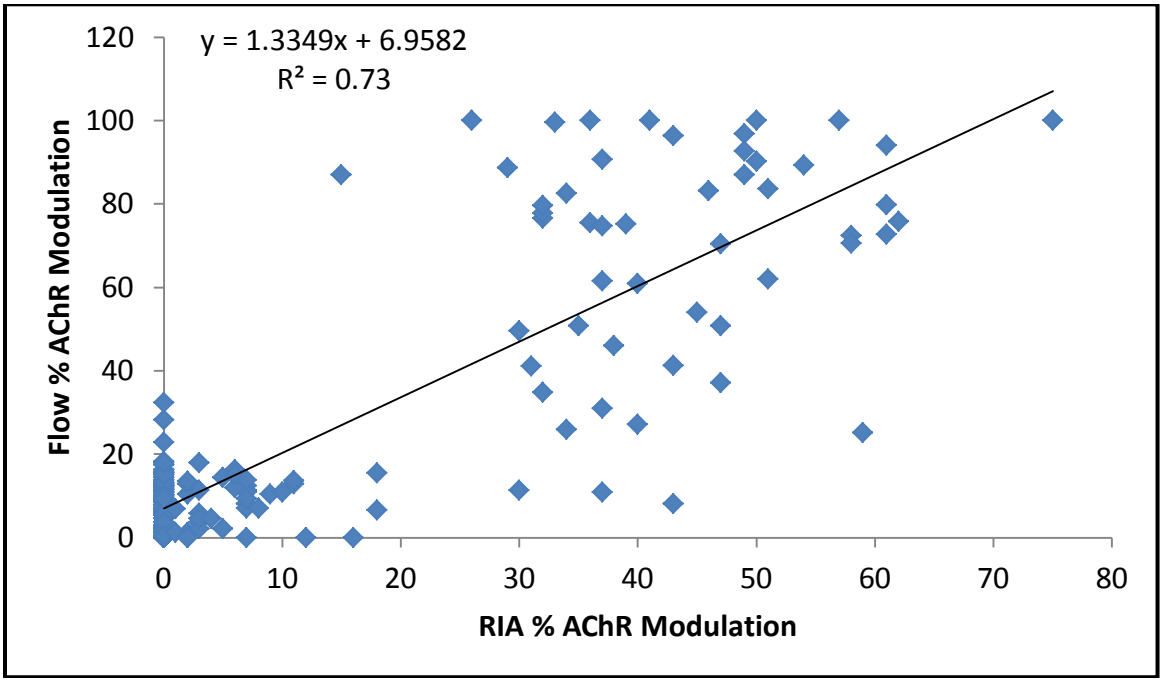

Figure 11. DB40 cell adherent-stained result correlation. Results correlation with the current assay. Results calculated using mean fluorescence of gated cells after background fluorescence normalization (Scheme B).

The ROC curve plot in Figure 9 shows an AUC value of 0.97, indicating very good sensitivity and specificity for adherent DB40 cell AChR modulating antibody results.

Figure 10 illustrates AChR modulating antibody result distribution for the DB40 cell line calculated using mean population fluorescence after background staining normalization (Scheme B). Positive cut-off for this calculation scheme was determined by ROC analysis to be 25% modulation or greater. Results for normal donor and AChR modulating antibody negative samples show little to no receptor modulation while the majority of the positive results show a high amount (>70%) modulation. Only three clinical positive samples resulted as false negative in relation to the current assay.

The linear regression plot in Figure 11 shows fair result correlation (R^2= 0.73) between results for DB40 cell Calculation Scheme B and those of the current assay.

Results for the linearity study are illustrated in Figure 12.

The AChR modulation response from the dilution series yielded strong responses for four two-fold dilutions. No dilution result fell below the 46% modulation positive cut-off value. Results from the dilution series were evaluated using the statistical program EP Evaluator and found to be nonlinear.

Results for analytical specificity are summarized in Table 3. Analytical specificity was found to be 100%. All AChR modulation results were negative for the samples tested.

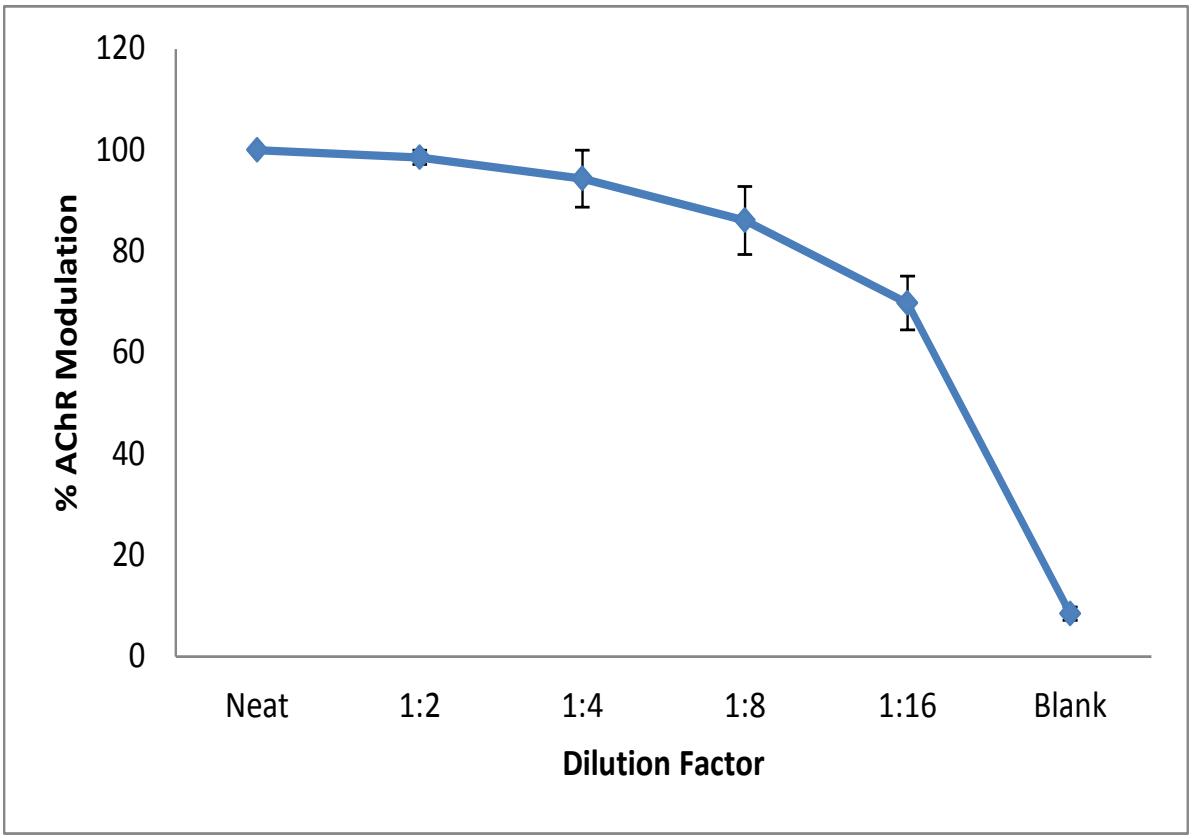

Figure 12. AChR modulating antibody positive sample dilution series.

Table 3. Results for analytical specificity

Sample Type	Clinical Result	% AChR Modulation by Flow
NMD Ab Positive	VGKC Pos	3
NMD Ab Equiv	VGKC +/=	3
NMD Ab Positive	VGKC Pos	0
NMD Ab Equiv	VGKC +/=	6
NMD Ab Positive	VGKC Pos	0
NMD Ab Positive	VGKC Pos	1
NMD Ab Positive	VGCC Pos	0
NMD Ab Positive	VGCC Pos	0
NMD Ab Positive	VGCC Pos	1
NMD Ab Positive	VGCC Pos	0
NMD Ab Positive	VGCC Pos	4
ANA Positive	1:160 Nucleolar	1
ANA Positive	1:320 Nucleolar	2
ANA Positive	1:320 Speckled	0
ANA Positive	1:160 Centromere	0
ANA Positive	1:160 Homogenous/Speckled	0
ANA Positive	1:2560 Centromere	0
ANA Positive	1:160 Homogenous	10
ANA Positive	1:1280 Speckled	6
ANA Positive	1:160 Homogenous/Speckled	22
ANA Positive	1:320 Homogenous	21

NMD Ab= Neuromuscular disease antibody. ANA=Anti-nuclear antibody. VGCC= Voltage-gated calcium channel antibody. VGKC= Voltage-gated potassium channel antibody. Reference range for AChR modulating antibody assay is <=45%

DISCUSSION

Due to the protocol used for the initial suspension-stained cell results, the cells tested experienced adverse conditions which negatively affected cellular integrity. These conditions were cold temperature and being in fluid suspension. This led to several assay failures and variable results (data not included) which brought the utility of this assay format into question. However, after several trials it became apparent how the RD cell could be made to perform consistently even under adverse conditions. The sample set used for the suspension-stain portion of the study was of note due to the fact that, as previously mentioned, 65 of the 118 clinical samples had discrepant AChR antibody test results (e.g., positive binding antibodies, negative modulating antibodies or vice versa). The remaining 53 clinical samples were either negative or positive for all available AChR antibody testing. It was encouraging to see that for samples that had discrepant modulating antibody flow cytometric and RIA results, the majority (26 of 33) were positive by the flow cytometric assay and had other positive AChR antibody results to corroborate. This suggests that the flow cytometric assay is more sensitive and robust, given that the AChR modulation effect was still demonstrated under adverse conditions. The suspension-stain flow results demonstrated lower sensitivity (91%) and agreement (84%) compared to the current assay due to inclusion of these discrepant results. However, when compared to the more sensitive AChR binding antibody assay, the current AChR modulating antibody radioimmunoassay shows 45% sensitivity while the

flow cytometric assay shows 66% sensitivity. Results for the normal donor samples show a lack of unexplained positive results suggesting good specificity. These observations suggest that the flow cytometric assay is a more sensitive, robust assay than the current format.

Experiences with staining RD cells in suspension made apparent the need to modify the assay format to improve cellular integrity prior to analysis. These modifications mostly consisted of performing the assay at room temperature without dissociating cells prior to staining. This resulted not only in improving cellular integrity but also greatly improving assay efficiency. The improved cellular integrity led to brighter cells due to better receptor retention in conjunction with use of the brighter APC fluorophore.

Samples used for the suspension-stain portion of the study were unavailable when testing commenced on the adherent-stain portion of the assay. The clinical samples tested in the second part of the study demonstrated more conventional result profiles, i.e., either positive or negative for all available AChR antibody testing. The use of these more conventional samples led to smaller differences in assay performance, but did not hinder evaluation.

The result calculation strategy in which background stain-normalized fluorescence was used (Calculation Scheme B) provided results that correlated most favorably with the current assay for both the RD and DB40 cell lines (Table 2). The two alternative calculation strategies seemed to either elevate results of normal donor and clinical negative samples or suppress results of clinical positive samples. The DB40 cell line demonstrated slightly lower sensitivity than the RD cell line, possibly due to defects

in receptor turnover or metabolism subsequent to gene transfection. It may be worthwhile to assess the effect of increased patient serum exposure concentration on the DB40 cell line as a means of increasing sensitivity in the future.

Results for the positive sample dilution series did not display linearity. Further investigation of a means of quantifying AChR modulating antibodies is not warranted based on these data. Due to the heterogeneous nature of autoantibody responses across patient populations combined with varying degrees of symptoms and serological results (12), it is reasonable to assume it would prove difficult to correlate the quantity of these antibodies with their effect.

Results for the analytical specificity showed no cross-reactivity in samples that tested positive for other neuromuscular disease antibodies (voltage-gated calcium and potassium channel antibodies) or for connective tissue disease (anti-nuclear) antibodies. These potential confounding conditions are not comprehensive, but results from these samples clearly suggest that interference from immune-mediated disorders other than myasthenia gravis is not likely.

CONCLUSION

Detection of AChR modulating antibodies by flow cytometry is a viable alternative to the current radioimmunoassay. Results from the RD cell line correlate best with the current assay when calculated using mean population fluorescence attenuated for nonspecific secondary antibody staining and inherent cell fluorescence. Of 170 samples tested, only 2 showed results that differed qualitatively with the current assay. One self-proclaimed healthy donor sample tested positive by the current modulating antibody assay and negative by the new modulating antibody assay (likely false positive). The other was an AChR antibody sample that tested negative by the current modulating antibody assay but positive by the new modulating antibody assay. This result was likely a false negative since the sample showed positive AChR binding and blocking antibody results to corroborate. Compared to the current radioimmunoassay, 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, use of the DB40 cell line for this application is not warranted.

This new assay format not only exceeds performance of the current assay, but also allows for the quantification of receptor expression on the RD cell facilitating more objective measurement of assay performance. This enhanced assay performance monitoring will likely lead to less run failures and delays in reporting clinical results due

to cell line failure. One last advantage of the new assay format is the ability to truly differentiate AChR modulating from AChR blocking antibodies. Both modulating and blocking antibody assay formats rely on ^{125}I α-bungarotoxin to label and quantify acetylcholine receptors, and AChR blocking antibodies can displace ^{125}I α-bungarotoxin leading to elevated AChR modulating antibody results. By labeling the Main Immunogenic Region of AChR α-subunits with a monoclonal antibody, the chance of erroneously decreased AChR levels is eliminated.

REFERENCES

1. Hughes T. The early history of myasthenia gravis. Neuromuscul Disord 2005;12:878-86.

2. Musilek K, Komloova K, Zavadova V, Holas O, Hrabinova M, Pohanka M, et al. Preparation and in vitro screening of symmetrical bispyridinium cholinesterase inhibitors bearing different connecting linkage-initial study for myasthenia gravis implications. Bioorg Med Chem Lett 2010;20:1763-6.

3. Juel VC, Massey JM. Myasthenia gravis. Orphanet J Rare Dis 2007;2:44.

4. Kuks JBM. Clinical presentation and epidemiology of myasthenia gravis. In: Kaminski HJ, editor. Myasthenia gravis and related disorders. 2nd Ed. New York: Humana Press; 2009. p. 79-94.

5. Tormoehlen LM, Pascuzzi RM. Thymoma, myasthenia gravis, and other paraneoplastic syndromes. Hematol Oncol Clin North Am 2008;22:509-26.

6. Jacob S, Viegas S, Lashley D, Hilton-Jones D. Myasthenia gravis and other neuromuscular junction disorders. Pract Neurol 2009;9:364-71.

7. Conti-Fine BM, Milani M, Kaminski HJ. Myasthenia gravis: past, present, and future. J Clin Invest 2006;116:2843-54.

8. Ubogu EE, Ruff RL. Neuromuscular junction physiology and pathophysiology. In: Kaminski HJ, editor. Myasthenia gravis and related disorders. 2nd Ed. New York: Humana Press; 2009. p. 1-12.

9. Lindstrom JM. Acetylcholine receptor structure. In: Kaminski HJ, editor. Myasthenia gravis and related disorders. 2nd Ed. New York: Humana Press; 2009. p. 13-41.

10. Newland CF, Beeson D, Vincent A, Newsom-Davis J. Functional and non-functional isoforms of the human muscle acetylcholine receptor. J Physiol 1995;489:767-78.

11. Meriggioli MN, Sanders DB. Autoimmune myasthenia gravis: emerging clinical and biological heterogeneity. Lancet Neurol 2009;8:475-90.

12. Conti-Fine BM, Diethelm-Okita B, Ostlie N, Wang W, Milani M. Immunopathogenesis of myasthenia gravis. In: Kaminski HJ, editor. Myasthenia gravis and related disorders. 2nd Ed. New York: Humana Press; 2009. p. 43-70.

13. Shiono H, Wong YL, Matthews I, et al. Spontaneous production of anti-IFN-alpha and anti-IL-12 autoantibodies by thymoma cells from myasthenia gravis patients suggests autoimmunization in the tumor. Int Immunol 2003;15:903-13.

14. Poëa-Guyon S, Christadoss P, Le Panse R, Guyon T, De Baets M, Wakkach A, et al. Effects of cytokines on acetylcholine receptor expression: implications for myasthenia gravis. J Immunol 2005;174:5941-9.

15. Strobel P, Chuang WY, Marx A. Thymoma-associated myasthenia gravis. In: Kaminski HJ, editor. Myasthenia gravis and related disorders. 2nd Ed. New York: Humana Press; 2009. p. 105-117.

16. Lanford JW, Phillips LH. Epidemiology and genetics of myasthenia gravis. In: Kaminski HJ, editor. Myasthenia gravis and related disorders. 2nd Ed. New York: Humana Press; 2009. p. 71-78.

17. Alshekhlee A, Miles JD, Katirji B, Preston DC, Kaminski HJ. Incidence and mortality rates of myasthenia gravis and myasthenic crisis in US hospitals. Neurology 2009;72:1548-54.

18. Agius MA, Richman DP, Vincent A. Autoantibody testing in the diagnosis and management of autoimmune disorders of neuromuscular transmission and related diseases. In: Kaminski HJ, editor. Myasthenia gravis and related disorders. 2nd Ed. New York: Humana Press; 2009. p. 143-156.

19. Keefe D, Hess D, Bosco J, Tzartos S, Powell J, Lamsa J, Josiah S. A rapid, fluorescence-based assay for detecting antigenic modulation of the acetylcholine receptor on human cell lines. Cytometry B Clin Cytom 2009;76:206-12.

20. Haven TR, Lyons BW, Pasi BM, Wu LL, Astill ME, Hill HR. Effect of acetylcholine receptor source on detection and quantification of anti-acetylcholine receptor-binding antibody [Letter]. Clin Chem 2008;54:1580-1.

21. Lyons BW, Wu LL, Astill ME, Wu JT. Development of an assay for modulating anti-acetylcholine receptor autoantibodies using human rhabdomyosarcoma cell line. J Clin Lab Anal 1998;12:315-9.

22. Benatar M. A systematic review of diagnostic studies in myasthenia gravis. Neuromuscul Disord 2006;16:459-67.

23. Massey JM. Acquired myasthenia gravis. Neurol Clin 1997;15:577-95.

24. Skeie GO, Apolstolski S, Evoli A, Gilhus NE,Illa I, Harms L, et al. Guidelines for treatment of autoimmune neuromuscular transmission disorders. Eur J Neurol 2010;17:893-902.

25. Haven TR, Astill ME, Pasi BM et al. An algorithm for acetylcholine receptor antibody testing in patients with suspected myasthenia gravis [Letter]. Clin Chem 2010 Jun;56:1028-9.