Assay Development and Measurement of Autoantibody-Mediated Complement Activity in Myasthenia Gravis

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ABSTRACT

A major subset of patients with myasthenia gravis (MG) harbor autoantibodies targeting the acetylcholine receptor (AChR) which can directly mediate neuromuscular junction (NMJ) damage through complement activation. Circulating AChR autoantibodies have highly heterogeneous properties that may influence their effector function capacity, including complement activity. In order to measure autoantibody-mediated complement activation in AChR MG patients and determine whether variable efficiency was observed, we developed a live cell-based assay (CBA) that measures AChR autoantibody–mediated complement effector function. The assay involved the expression of AChR on a modified HEK cell line in which the complement regulator genes (CD46, CD55, and CD59) had been knocked out. AChR autoantibody–mediated complement activity was measured using flow cytometry by specifically detecting the membrane attack complex (MAC), the terminal protein assembly in the complement cascade. An association between MAC formation and disease severity as measured by the MGFA classification was found, as well as between autoantibody-mediated complement activity and autoantibody titer. However, outlying samples that included high AChR binders with low complement activity as well as low AChR binders with high complement activity were observed. This mini-review of our previously reported study focuses on complement assay development and the heterogeneity in AChR autoantibody-mediated complement activation.

Introduction

A fundamental pathogenic mechanism of myasthenia gravis (MG) is the activation of complement by acetylcholine receptor (AChR) autoantibodies (1-3). Consequently, this mechanism is a sound target for therapeutic intervention. Indeed, therapeutics that target AChR autoantibody-mediated complement activity limit the capacity of autoantibodies to damage the postsynaptic muscle membrane. Specifically, eculizumab, an anti-C5 monoclonal, and zilucoplan, a peptide, bind to C5 and thereby inhibit C5 cleavage to C5a and C5b and the subsequent generation of the terminal complement complex, C5b-9. Both therapeutics provide benefit to AChR MG patients (4-7). For example, phase III clinical trials of eculizumab have shown efficacy in well over half of treated patients. Unfortunately, 40% of patients did not meet the trial endpoint and some required rescue therapy (5, 6).

The poor responders had measurable circulating AChR autoantibodies, but the titer of the autoantibodies did not associate with response. Given that AChR autoantibodies were present, and a key mechanism of their pathology is complement activation, the trial outcome presents a challenging reconciliation. These results also highlight the limitations of using AChR autoantibody titer as a biomarker. Importantly, it emphasizes the need for further understanding of the variability in AChR autoantibody–mediated pathogenic mechanisms so that the response to treatments can be better anticipated. This mini-review of our work presented at the 14th Myasthenia Gravis Foundation of American (MGFA) International Conference, is focused on describing the development of a novel assay for investigating AChR autoantibody-mediated complement activity, and understanding the observed heterogeneity underlying autoantibody-mediated pathogenic mechanisms in MG.

AChR autoantibody pathogenic mechanisms

AChR autoantibodies elicit tissue damage through three distinct mechanisms (**Figure 1A**) (8-18). The first is receptor internalization (often termed modulation) of AChR, which occurs when an autoantibody divalently binds to two adjacent AChR molecules, causing the cross-linked AChR to be internalized via endocytosis, leading to its degradation. This ultimately reduces the number of AChR molecules present on the cell surface and leads to reduction in neuromuscular transmission. The second mechanism is receptor blocking where autoantibodies prevent acetylcholine (ACh) from binding to AChR by binding close to, or at, the ACh binding site. When ACh is impaired from binding to AChR, the flow of ions across the cell membrane is inhibited (14, 18). It is also reasonable to consider that some blocking antibodies, which do not bind specifically at the ACh binding site, may nonetheless inhibit signaling by altering the conformational state of the AChR such that ACh binding is inefficient. The third mechanism is complement activation, where AChR autoantibodies activate the classical complement pathway (6, 14, 19, 20). The pathway is initiated by the binding of C1q, a component of the complement pathway, to the Fc region of an antibody. This binding promotes subsequent proteolysis of precursor complement proteins that eventually leads to the formation of a membrane attack complex (MAC) or terminal complement complex (TCC). The MAC can cause destruction of the cell membrane, which causes cell death through lysis (21) (**Figure 1B**).

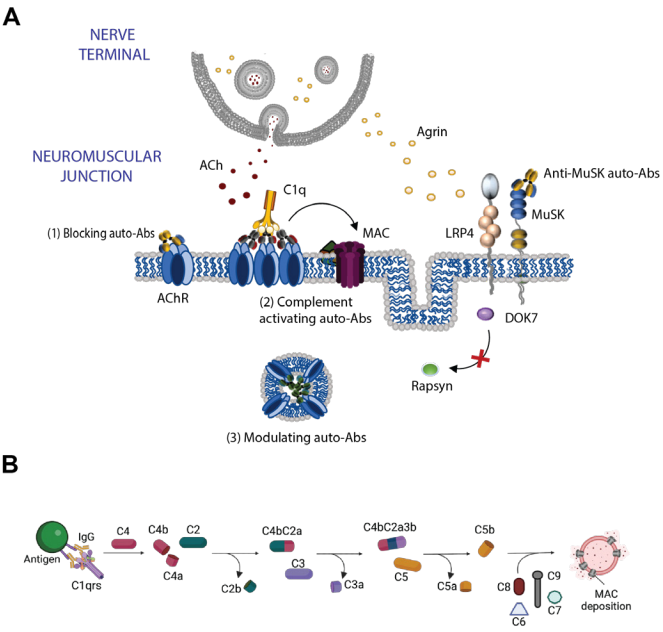

Figure 1. Autoantibody-mediated mechanisms of MG pathology at the neuromuscular junction (NMJ)

A. In a normal NMJ, action potential at the presynaptic nerve terminal releases acetylcholine (ACh) and agrin into the synaptic cleft. ACh binds to acetylcholine receptor (AChR) triggering ion flux and subsequently muscle contraction. Agrin binds to LRP4 leading to MuSK phosphorylation and DOK7 recruitment and rapsyn activation. This leads to AChR clustering and NMJ integrity. In MG, autoantibodies disrupt the NMJ structural integrity and/or neurotransmission. AChR autoantibodies interfere with AChR signaling via (1) blocking ACh, (2) initiating the complement cascade or (3) modulating/internalizing AChR. Anti-MuSK autoantibodies hinder agrin-LRP4-MuSK interaction, thus obstructing AChR clustering, causing reduced clustering and decrease in junctional folds and neuromuscular transmission.
B. The classical complement pathway is activated via C1q binding to an antigen-antibody complex. Following activation, a cascade of protein lysis is initiated that leads to the generation of C3 convertase (C4b2a), which cleaves C3. Following C3 cleavage into C3a and C3b, C3b binds to C4bC2a to generate C5 convertase (C4b2a3b), which initiates the assembly of the membrane attack complex (MAC). The MAC induces cell lysis and death via disruption of the target cell membrane. *NMJ: neuromuscular junction; ACh: acetylcholine; AChR: acetylcholine receptor, MuSK: muscle-specific kinase; LRP4: low-density lipoprotein receptor-related protein 4*

The relative distribution of AChR autoantibodies capable of one (or more than one) of these pathogenic mechanisms in individual patients is not well understood. It is likely to considerably differ between patients and may fluctuate within patients over time and in response to treatment. A means to measure the frequency of unique AChR autoantibody-mediated mechanisms may help in predicting patient response to treatments, especially complement inhibitors. Thus, we sought to examine the relative contribution of the autoantibody-mediated complement mechanism present in serum samples from individual patients. To that end, we adapted the highly sensitive live cell-based assay (CBA) (22), which is usually used to measure AChR autoantibody binding, to quantify autoantibody-mediated complement activation.

Development of an assay to measure AChR autoantibody-mediated complement activation.

Cell-based assays (CBAs) constitute a sensitive method for detecting serum autoantibodies in AChR MG patients. The assay utilizes HEK cells that transiently express the four subunits of the adult AChR receptor, along with rapsyn-green fluorescent protein (GFP) to promote receptor clustering and detection of transfected cells. In the CBA, the native pentameric complex of AChR retains its native structure, whereas other assay formats may disrupt antigen epitopes due to solubilization reagents, purification approaches or antigen immobilization. Furthermore, the CBA allows for the transfection of accessory proteins, which provides a better representation of the *in vivo* NMJ environment. Specifically, the co-expression of the scaffolding protein, rapsyn, with AChR in HEK cells results in the clustering of AChR on the cell surface. This leads to increased assay sensitivity, which was a key development (22) in the detection of AChR autoantibodies in a subset of MG patients thought to be seronegative. These findings has been subsequently confirmed in other independent studies (23, 24).

Accordingly, this platform was leveraged to develop an assay that can measure autoantibody-mediated complement activation (**Figure 2A**). Initial attempts to observe complement assembly on the AChR transfected cells failed. Interestingly, this result stood in stark contrast to what we observed with other autoantigens, including aquaporin-4 (AQP4) and myelin oligodendrocyte glycoprotein (MOG), the targets of autoantibodies found respectively in neuromyelitis optica spectrum disorder (NMOSD) and myelin oligodendrocyte glycoprotein antibody disease (MOGAD). Here, robust complement assembly—mediated by AQP4 and MOG autoantibodies—was readily detectable (25). To address this obstacle, we turned to a previous study that demonstrated increased complement component deposition by disruption of complement regulator/inhibitor expression (26). Accordingly, the genes for the mammalian

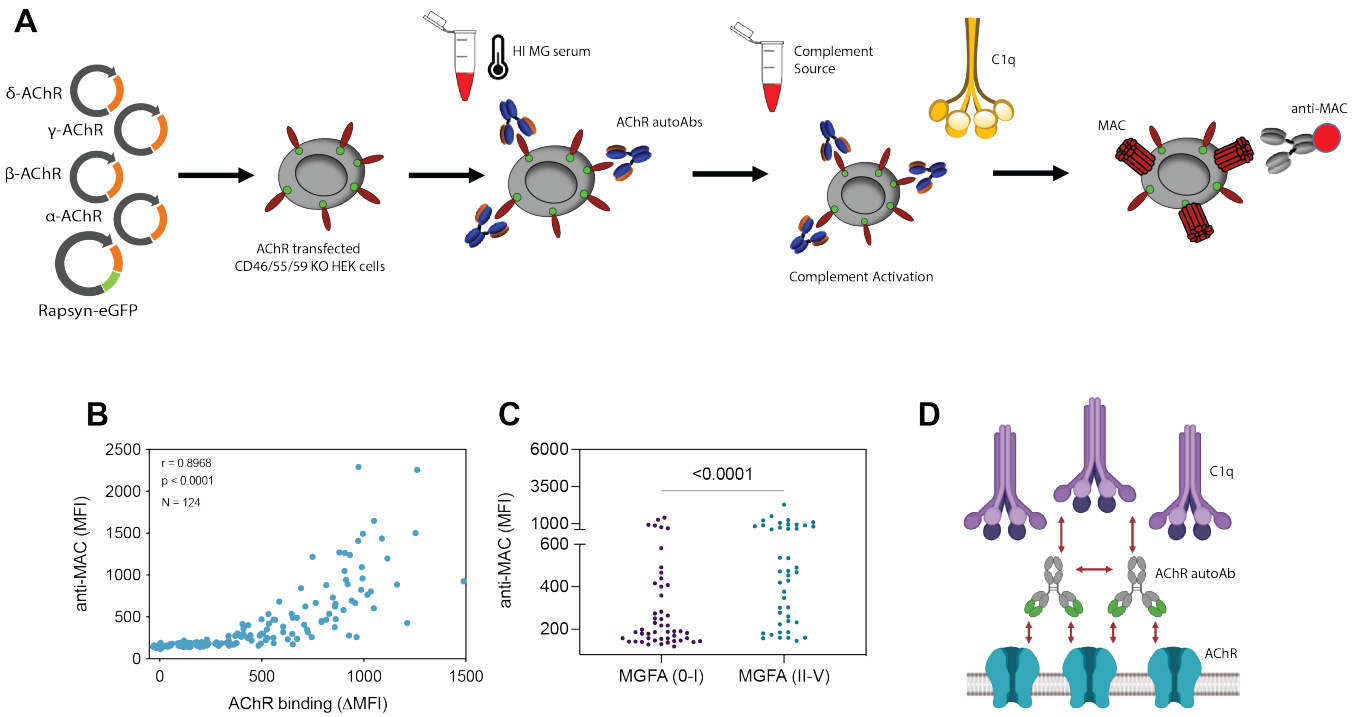

Figure 2. Heterogeneity in Autoantibody-Mediated Complement Activity

A. Schematic of our complement cell-based assay. CD46/55/59 knockout HEK cells are transfected with AChR subunits and rapsyn to express clustered AChR at the cell surface. This is followed by the application of heat-inactivated patient serum and the addition of a consistent complement protein source. MAC formation is measured via staining with anti-MAC antibody and visualization with flow cytometry. **B.** Correlation between autoantibody-mediated MAC formation and AChR binding in AChR MG patients (r=0.8968, p<0.0001). **C.** Differences in MAC formation between samples with low disease severity (MGFA 0/I) and higher disease severity (MGFA II-V). Samples showed a median MAC mean fluorescent intensity (MFI) of 190.3 in samples with MGFA 0-I compared to 468.3 in samples with MGFA II-V (p-value <0.0001). **D.** Schematic of the interactions that support optimum complement activation, which include epitope binding site, spatial arrangement of target antigen, minimum steric interference in the Fc-Fc interactions at the CH3 domain, and C1q-Fc interactions at the CH2 domain. The plots shown in **B** and **C** were constructed from our previously published data (Neurol Neuroimmunol Neuroinflamm. 2022 doi: 10.1212/NXI.0000000000001169. PMID: 35473886)

complement inhibitors CD46, CD55, and CD59 were knocked out in HEK cells using the CRISPR/Cas9 system. Using the modified HEK cells afforded a functional assay to effectively measure AChR autoantibody-dependent complement fixation. Intriguingly, another group also favored the use of a CD46, CD55, and CD59 triple knockout ARPE19 cell line to develop an in vitro assay that allows for testing autoantibody complement activation. They also transfected cells with plasmids encoding AChR subunits and rapsyn, and utilized pooled human serum as a source of complement (27).

It is unclear why measurement of AChR autoantibody-dependent complement fixation required the absence of the CD46, CD55, and CD59 complement regulators, unlike fixation mediated by MOG and AQP4 autoantibodies. However, these regulators have previously been observed to influence MG immunopathology. For example, CD55 knockout mice were shown to be more susceptible to the effects of pathogenic MG autoantibodies (19, 28, 29). In other MG experimental models (mice and rats), complement inhibition has shown efficacy in reducing the effects of the autoantibody response generated by the injection of AChR or peptide fragments of AChR (30, 31). Finally, the extraocular muscle subgroups are highly associated with MG. Interestingly, they express reduced levels of CD55 and CD59, suggesting that diminished complement regulatory activity may contribute to the susceptibility of these muscle groups in MG (19).

During the development of the assay, we also considered how MAC-dependent cell death might influence sensitivity while the assay is being performed, given that the cells must be intact and alive to be measured accurately by FACS. To improve sensitivity, we considered Cr^{52} release to measure cumulative cell death, but we were reluctant to introduce radioactivity into the assay. Instead, we tested an alternative approach with our MOG autoantibody assay to address this concern (25): autoantibody-dependent complement is activated but arrested prior to MAC formation, thus

avoiding cell death. Specifically, a human complement source depleted of C8—a requirement for MAC formation—was used. Complement activity was measured using an antibody specific for C3d (32) which covalently attaches to target cells upon complement initiation. While C3d deposition was detected, no conspicuous increase in sensitivity was observed.

An alternative approach to measuring AChR autoantibody-mediated complement fixation was recently developed which may address some limitations of the CBA approach. This bioassay leveraged intact innervated muscle tissue (33). Here, the authors developed a sophisticated assay that facilitates the visualization of the NMJ using mouse diaphragm-phrenic nerve preparations with physiologically normal characteristics. This methodology eliminates the issues associated with the removal of the complement inhibitory proteins and more accurately reflects the NMJ as it ensures proper density and clustering of AChR. Nevertheless, this approach requires time and resources that does not—at this early stage of its development—allow for the high throughput evaluation of large patient cohorts.

Measuring AChR autoantibody-mediated complement activation in patient serum.

We next used our assay to analyze serum samples from a cohort of MG patients. The assay showed that autoantibody binding was highly correlated with MAC formation (**Figure 2B**). However, heterogeneity was found in the patient cohort, where some cross sectional and longitudinal patients had high AChR autoantibody titers but low complement activity, while others had low titer but high complement activity. These findings suggest that while the majority of AChR autoantibodies can cause tissue damage through complement activation, binding alone does not dictate MAC formation. This was further highlighted when the association between complement deposition and disease severity was examined, and a modest correlation between MAC formation and MGFA classification was found (**Figure 2C**). However, heterogeneity was also observed where there were patients that had high disease severity but low MAC formation while others had relatively elevated MAC formation, but low disease severity scores.

The differences in MAC formation in two subsets of MG, namely early-onset MG (EOMG) and late-onset MG (LOMG), were investigated. No significant differences were observed, which may suggest that there are no major variations in the complement associated properties of the AChR autoantibodies found in the two MG subtypes. Furthermore, there were no differences in MAC formation in patients who had immune modulatory therapy or thymectomy. Given that AChR autoantibodies persist after these treatments (34, 35), it is plausible to conclude that these treatments have minimal effect on the ability of existing autoantibodies to mediate complement activity.

Understanding the heterogeneity of AChR autoantibody-mediated complement activity.

The heterogeneity that we observed in the efficiency of AChR autoantibody-mediated complement activation point to the complexity of the autoantibody repertoire in AChR MG. Patients may harbor AChR autoantibodies; however, whether they mediate MAC formation that contribute to disease severity is subject to multiple factors. These factors may include whether they are tissue resident or in circulation, patient genetics, and the expression levels of complement inhibitors on the muscle tissue. Furthermore, AChR autoantibodies may elicit pathogenicity through other mechanisms, such as blocking of ACh or modulation/internalization of AChR, which results in reduction in neuromuscular transmission. It is also possible that patients with high binding, but low disease severity may have autoantibodies that bind to AChR without effectively causing any tissue damage. The presence of such putative 'binding only' autoantibodies have been reported in autoimmune disorders such as pemphigus (36) and NMO (37).

The disassociation between AChR autoantibody titer and disease severity highlights the complexity of their pathogenic properties. While the detection of circulating AChR autoantibodies can confirm MG diagnosis, the titers can vary widely among individuals and during disease progression. Some patients with a mild phenotype can have very high AChR autoantibody titers, while others with severe disease during a relapse can have very low titers (38-42). Though changes of titer within an individual can be associated with disease severity, it is often observed that AChR autoantibody titer measured at a single point does not correlate well with disease severity or activity and makes it difficult to use titer as a reliable biomarker. The disparity between disease severity and titer may be explained—in part—by the inability of clinical assays to distinguish between AChR autoantibody titer and pathogenic mechanisms.

In addition to variable titers, circulating AChR autoantibodies have highly heterogeneous binding properties that may influence their effector functions. Adult AChR is a pentameric structure consisting of 2α: β: ε: δ subunits while fetal AChR has a similar structure where there is a gamma in lieu of an epsilon subunit (2α: β: γ: δ) (43). AChR autoantibodies are polyclonal in nature; they can bind any of the AChR subunits and various epitopes present on each subunit. The majority of serum AChR autoantibodies bind to the main immunogenic region (MIR) that resides primarily, *but not exclusively*, on the alpha subunit (44, 45); however robust binding to other subunits has also been observed (46).

It is likely that AChR autoantibodies with different subunit and/or epitope targets vary in their efficiency at activating complement. The relationship between epitope binding specificity and complement activation has been

elegantly demonstrated for AQP4-binding autoantibodies. Specifically, AQP4 autoantibody binding alone is not sufficient to induce complement-mediated cell death (47). Instead specific epitope binding and the assembly of multimeric platforms are necessary for optimum complement-mediated cell death (**Figure 2D**)(47). AQP4 autoantibodies that bind epitopes on the extracellular loop C display significantly higher complement activity compared to autoantibodies that target other epitopes. Moreover, AQP4 forms supramolecular orthogonal arrays that organize these epitopes in a manner that enhances the formation of autoantibody multimeric complexes through Fc-Fc interactions and efficient C1q binding, resulting in optimized complement activation (47). In the context of MG, it has been proposed that combinations of recombinant monoclonal antibodies that target specific subunits of AChR increased complement activation in vitro and in a passive transfer-based MG animal model (48). Here, it was hypothesized that the formation of larger AChR clusters and enhanced Fc-Fc interactions increased the magnitude of the autoantibody-mediated complement activation (48). Continued studies of human derived, monoclonal AChR autoantibodies (48, 49) to further understand the relationship between autoantibody binding properties and their effector functions will be necessary to understand these relationships with more granularity.

In addition to binding properties mediated by the variable region of antibodies, the constant region, namely the Fc, can influence effector functions including complement (50, 51). Differences in Fc regions are observed due to IgG subclass usage, constant region polymorphisms, varying glycosylation patterns and post-translational modifications (52, 53). Complement activation is influenced by IgG subclass where IgG3/ IgG1 demonstrates the greatest activation while IgG4 demonstrates negligible activity (54). Furthermore, post-translation modification (PTM) can alter the structure and stability of an antibody as well as its capability to activate complement (50). The IgG Fc domain includes a highly conserved glycosylation site in the constant heavy chain 2 (CH2) domain. Carbohydrate moieties attached to this site can influence the interactions between an antibody and complement proteins. This has been observed in MOGAD, where higher inflammatory profiles were associated with an increase in agalactosylated and asialylated glycovariants on IgGs (55). Furthermore, sialyation of the site can also decrease inflammatory responses by interfering with complement-mediated cytotoxicity (51). The interplay between all these variables can have a major effect on how these autoantibodies elicit tissue damage and understanding this complexity in AChR MG may help develop precisely targeted and personalized therapies.

Conclusions

To understand MG disease course heterogeneity more deeply, future efforts should include the development and application of assays that can accurately measure the composition of the AChR autoantibody repertoire and the varying pathogenic mechanisms they can mediate. These assays should ideally include measures of binding-only, classical pathway complement activation, as well as modulating and blocking functions. Collectively, these measurements may provide valuable insights into disease progression and serve as an improved biomarker for MG compared to autoantibody binding alone. By targeting unique autoantibody-mediated pathogenic pathways, clinicians may be able to develop more individualized and effective treatment plans for their patients.

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Study funding:
KCO was supported by the National Institute of Allergy and Infectious Diseases of the NIH under award numbers R01-AI114780 and R21 AI164590; through an MGNet pilot grant awarded through the Rare Diseases Clinical Research Consortia of the NIH (award number U54-NS115054) and by a High Impact Clinical Research and Scientific Pilot Project award from the Myasthenia Gravis Foundation of American (MGFA).

Financial disclosures:
Abeer H. Obaid reports no disclosures.

Relevant to this work, Dr. Kevin C. O'Connor has received research support from Ra Pharma, now a part of UCB Pharma, and Alexion, now part of AstraZeneca. KCO is a consultant and equity shareholder of Cabaletta Bio. KCO has served as consultant/advisor for Alexion Pharmaceuticals, now part of AstraZeneca.

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