

Comparison of Fixed and Live Cell-Based Assay for the Detection of AChR and MuSK Antibodies in Myasthenia Gravis

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Abstract

Background and Objectives

Live cell-based assay (CBA) can detect acetylcholine receptors (AChRs) or muscle-specific tyrosine kinase (MuSK) antibodies (Abs) in a proportion of patients with radioimmunoassay (RIA)-double seronegative myasthenia gravis (dSN-MG). A commercial fixed CBA for AChR and MuSK Abs has recently become available; however, comparative studies on fixed and live CBAs are lacking. In this study, we compared the performance of fixed and live CBAs in patients with RIA-dSN MG and assessed their sensitivity in RIA-positive MG samples and their specificity.

Methods

AChR and MuSK Abs were tested in 292 serum samples from 2 Italian MG referral centers by live and fixed CBAs: 192 from patients with MG and 100 from controls. All samples had been previously assessed by RIA: 66 were AChR positive, 40 MuSK positive, and 86 dSN. All controls were negative. Two independent raters assessed the CBA results. Fixed and live CBAs were compared with the McNemar test; interrater and interlaboratory agreement were assessed with Cohen's kappa or interclass correlation coefficient (ICC), as appropriate.

Results

In 86 RIA-dSN samples, fixed CBA detected Abs in 10 cases (11.6%, 95% CI 5.7–20.3), whereas live CBA detected Abs in 16 (18.6%, 95% CI 11.0–28.5) ($p = 0.0143$). Of these sera, those positive by fixed CBA were also positive by live CBA. In addition, live CBA could detect MuSK Abs in 4 and AChR Abs in 2 samples that were negative by fixed CBA, providing an 8% (95% CI 2.9–16.6) further increase in the Ab detection rate. These results were confirmed by flow cytometry. In the RIA-positive cohort, the sensitivity for AChR Abs was 98.5% (95% CI 91.9%–99.9%) for fixed CBA and 100% (95% CI 94.6–100) for live CBA ($p = 0.1573$). For both assays, the sensitivity for MuSK Abs was 100% (95% CI 91.2–100), and the specificity was 100% (95% CI 96.4–100). Interrater agreement was almost perfect for live and fixed CBAs (Cohen's kappa 0.972 and 0.978, respectively), alike interlaboratory agreement. Interrater agreement for the CBA score ranged from good to excellent (ICC: 0.832–0.973).

Discussion

Fixed CBA represents a valuable alternative to RIA for AChR and MuSK Ab detection in patients with MG and could be considered as a first-step diagnostic test. Live CBA can be useful in the serologic evaluation of RIA- and fixed CBA-negative samples.

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Glossary

Ab = antibody; **AChR** = acetylcholine receptor; **CBA** = cell-based assay; **dMFI** = delta median fluorescent intensity; **dSN** = double seronegative; **eGFP** = enhanced green fluorescent protein; **FACS** = fluorescence-activated cell sorting; **HEK293** = human embryonic kidney cells 293; **ICC** = interclass correlation coefficient; **MG** = myasthenia gravis; **MuSK** = muscle-specific tyrosine kinase; **RIA** = radioimmunoassay; **UCSC** = Università Cattolica del Sacro Cuore.

Myasthenia gravis (MG) is an autoimmune disorder caused by antibodies (Abs) targeting different proteins at the neuromuscular junction.¹ When MG is suspected on clinical grounds, Ab detection confirms the diagnosis and provides the rationale for patient subgrouping and personalized treatment. Radioimmunoassay (RIA), the current gold standard for Ab identification in MG, can detect Abs to the nicotinic acetylcholine receptor (AChR) in 80%–85% and to the muscle-specific tyrosine kinase (MuSK) in 5%–7% of patients with MG.^{2,3} About 10%–15% of patients with MG have no detectable Abs by RIA and are referred to as double seronegative MG (RIA-dSN MG), although live cell-based assays (CBAs) can detect low-affinity Abs to clustered adult and fetal AChR or MuSK in a proportion of these patients.^{4,8} Live CBA using human embryonic kidney (HEK293) cells transfected to overexpress the clustered AChR isoforms,^{4,6,8} or MuSK,^{7,8} on the cell surface as in vivo, not only proved to be more sensitive than RIA but also highly specific.^{4,7,8} Indeed, the high-density expression of the antigen in its native conformation and the advantage of cotransfecting the antigen with rapsyn to maintain AChR clustering on the cell surface facilitate the binding of low-affinity AChR Abs.⁹ This advance in the Ab testing allowed a significant improvement in the serologic diagnosis of MG, as recently confirmed by a study from our group finding clustered AChR or MuSK Abs in around one-third of patients with RIA-dSN MG.⁸ However, the use of live CBA is currently limited to specialized research settings because it requires expertise and cell-culture facilities. A commercial fixed CBA for adult and fetal AChR and MuSK Ab detection has recently become available, widening the possibility to improve MG serologic diagnosis and providing a reliable test without the need for radioactive ligands.⁸ Recently, the performance of fixed CBA for AChR Ab detection was compared with RIA (cutoff for positivity 0.5 nmol/L), showing 4% increased sensitivity with 99% specificity.¹⁰ To date, there are no studies comparing fixed and live CBAs, which could clarify their respective role in the MG diagnostic algorithm. The aims of this study were to (1) compare the performance of fixed and live CBAs in the detection of AChR and MuSK Abs in patients with RIA-dSN MG and (2) assess their sensitivity in RIA-positive samples and their specificity in healthy and neurologic disease controls.

Methods

Sample Selection

In this retrospective study, we tested a total number of 292 serum samples, including 192 sera from patients with MG (86 RIA-dSN, 66 AChR RIA positive, and 40 MuSK RIA positive)

and 100 from controls. In all samples, RIA was performed by commercial assay (RSR Limited, Cardiff, UK; cutoff value for positivity ≥ 0.5 nMol/L for AChR Abs and ≥ 0.05 nMol/L for MuSK Abs); all control sera tested negative. MG samples were obtained from 2 Italian MG referral centers, Policlinico Gemelli IRCCS—Università Cattolica del Sacro Cuore (UCSC) (n = 146) and IRCCS Mondino Foundation, Pavia (n = 46). We included the earliest available sera from RIA-dSN patients. AChR and MuSK RIA-positive samples were randomly selected from our sera biobanks. In addition, we tested the serum samples from 40 healthy subjects and 60 patients with other neurologic disorders (inflammatory neuropathy, 30; amyotrophic lateral sclerosis, 17; multiple sclerosis, 10; and paraneoplastic neuropathies, 3). In RIA-dSN patients, MG diagnosis was based on typical history and signs plus neuromuscular transmission impairment on repetitive nerve stimulation or single-fiber EMG.¹¹

Antibody Detection

At the time of the study, all sera were retested by RIA (RSR Limited, Cardiff, United Kingdom) to confirm the previous results and to rule out Ab degradation of stored samples. Sera were kept at -20°C during the study period, while stored at -80°C for longer periods. RIA was performed exclusively in the laboratory of UCSC, Rome, and blindly assessed by 2 raters (C.C. and G.M.). Afterward, all sera were tested for Abs to the adult/fetal AChR and MuSK with an in-house live CBA and with the commercial fixed CBA (Euroimmun, Lubeck, Germany).⁸ Samples were tested in the laboratories of the 2 institutions (n = 206 UCSC, Rome, Italy; n = 86 IRCCS Mondino Foundation, Pavia, Italy), using the same protocols briefly described as follows. Fixed CBA was performed according to the manufacturer's instructions (serum dilution 1:10). For the live CBA, HEK293 cells were transiently transfected to express AChR, clustered through the cotransfection with enhanced green fluorescent protein (eGFP)-conjugated rapsyn or MuSK-eGFP, and 48 hours after transfection, live cells were incubated with sera (1:20 dilution), as previously described.^{4,7} Human IgG labeling was demonstrated with a secondary goat anti-human IgG Fc-specific unconjugated cross-adsorbed Ab (1:750, ref# 31125, Invitrogen), followed by a tertiary donkey anti-goat IgG (H + L) AF568-conjugated Ab (1:750, ref# A11057, Invitrogen). CBA results were evaluated through a fluorescence microscope by 2 independent raters (G.S. and V.D., Policlinico Gemelli IRCCS, Roma; M.G. and S.S., IRCCS Mondino Foundation, Pavia) blinded to the results of the other assays and clinical information. Labeling of the secondary antibodies was scored by 2 independent blinded observers as previously

described: (0) = no labeling; (1) = weak labeling of some transfected cells; (2) = moderate labeling of approximately 20%–50% of transfected cells; (3) = moderate/strong labeling of approximately 50%–80% of transfected cells; and (4) = strong labeling of almost all transfected cells.⁴ Samples with a CBA score of ≥ 1 were considered positive.⁴ For the intralaboratory interrater agreement, we assessed both the dichotomous assay result and the CBA score. We used the assays' dichotomous result (positive vs negative) for the comparison between live and fixed CBAs and for the interlaboratory agreement. To perform the latter, 96 serum samples (56 from patients with MG and 40 from controls) were tested in both centers. All discrepancies between raters were solved on blinded re-evaluation of the assay, and if no consensus was reached, samples were retested. In sera ($n = 2$) with unsolved discrepancies, samples were considered positive if they were assessed as such by at least one of the independent raters. Discrepancies in the CBA scores were addressed first through blinded re-evaluation of the assays and second through unblinded re-evaluation; in cases of unsolved discrepancies, the higher CBA score was considered for data analysis and graph building.

To confirm the Ab labeling to adult/fetal AChR isoform or MuSK, we further developed the live CBA using fluorescence-activated cell sorting (FACS) analysis, by which we tested a proportion of the study samples ($n = 234/292$, 167 from patients with MG, 30 from healthy controls, and 37 from disease controls). Briefly, all sera were incubated with AChR-eGFP- or MuSK-eGFP-transfected HEK293 cells in suspension, and after washing, IgG binding was detected with a secondary goat anti-human IgG Fc-specific unconjugated cross-adsorbed Ab (1:200, ref. 31125, Invitrogen), followed by a tertiary donkey anti-goat IgG (H + L) AF568-conjugated Ab (1:200, ref# A11057, Invitrogen, US) using CytoFLEX flow cytometer. The AChR-IgG level was calculated by the delta median fluorescent intensity (dMFI) of the transfected (single cells/viable/eGFP-positive gates) minus untransfected (single cells/viable/eGFP-negative gates) cells. The cutoff was determined for each dMFI using 10 healthy controls (mean value plus 3 SDs). FACS assays were performed at UCSC laboratory.

Statistical Analysis

Continuous variables were presented as median with interquartile range (IQR) and categorical variables as proportions and percentages. The CBA Ab detection rate in RIA-dSN MG was calculated as the proportion (with 95% CI) of CBA-positive samples in the RIA-negative patient cohort. CBA sensitivity (with 95% CI) was assessed in the RIA-positive cohort, and CBA specificity (with 95% CI) was calculated as the proportion of CBA-negative samples in the healthy and disease control cohorts. The results (positive/negative) of fixed CBA and live CBA were compared with the McNemar test. Interrater agreement was evaluated with Cohen's kappa when the assay result was categorical (positive/negative) and with the interclass correlation coefficient (ICC) when it was

continuous (CBA score). Interlaboratory agreement was assessed with Cohen's kappa. Statistical analysis was performed with STATA v 16 (STATA Corp, College Station, TX).

Standard Protocol Approvals, Registrations, and Patient Consents

All patients provided written consent to the use of their serum samples (taken for diagnostic purposes) in this study. Healthy controls provided written consent. The study was conducted according to the Helsinki Declaration and approved by the Ethics Committee of the UCSC (Rome, Italy) with E.C. protocol number 0008363/21 and of the IRCCS Policlinico San Matteo (Pavia, Italy) with E.C. protocol number P-20170027756.

Data Availability

Anonymized data not published within this article will be made available on reasonable request from any qualified investigator.

Results

Patients with RIA-dSN MG had a median age of 43 years (IQR: 32–55) at disease onset; 46 of 86 (53%) were females, and 52 of 86 (61%) cases had a generalized disease course. Forty of 86 (47%) patients were immunotherapy naive at sampling. Among RIA-positive patients, the median age at MG onset was 59 years (IQR: 39–67) for AChR-positive patients and 34.5 years (IQR: 26–51) for MuSK-positive patients; the female proportion was 24 of 66 (36%) and 29 of 40 (74%) patients, respectively. Detailed clinical information is reported in eTable 1 (links.lww.com/NXI/A749). The CBA results are summarized in Table 1 and are presented in eFigures 1 and 2 (links.lww.com/NXI/A749).

Among RIA-dSN patients ($n = 86$), fixed CBA detected Abs in 10 of 86 cases (11.6%, 95% CI 5.7–20.3, Figure 1, A–C) and live CBA in 16 of 86 samples (18.6%, 95% CI 11.0–28.5; Figure 1, D–L) ($p = 0.0143$). In particular, fixed CBA detected AChR Abs in 9 of 86 (10.5%, 95% CI 4.9–18.9) and MuSK Abs in 1 of 86 (1.2%, 95% CI 0.03–6.3) cases, whereas AChR Abs were found by live CBA in 11 of 86 cases (12.8%, 95% CI 6.6–21.7; $p = 0.1573$) and MuSK Abs in 5 of 86 (5.8%, 95% CI 1.9–13.1; $p = 0.0455$). Of these sera, those positive by fixed CBA were also positive by live CBA (eFigure 1, links.lww.com/NXI/A749). In addition, the latter could detect MuSK Abs in 4 samples and AChR Abs in 2 sera that were negative by fixed CBA, providing an 8% (95% CI 2.9–16.6) increase in the Ab detection rate. No patient was double positive for AChR and MuSK Abs by RIA, fixed CBA, or live CBA. RIA-dSN CBA-positive patients ($n = 16$) had a median age at disease onset of 34 years (IQR: 27–60.5); 7 (44%) were females, with a purely ocular involvement in half of the cases and concomitant autoimmune comorbidities in 3 (19%; thyroiditis, 2; vitiligo, 1). Demographic and clinical data of RIA-dSN CBA-positive patients are reported in eTable 2 (links.lww.com/NXI/A749).

Table 1 Cell-Based Assay Metrics

	Commercial fixed CBA	In-house live CBA	p Value
RIA-dSN MG cohort (N = 86)			
Ab detection rate (95% CI), %	11.6 (5.7–20.3)	18.6 (11.0–28.5)	0.0143
AChR Ab detection rate (95% CI), %	10.5 (4.9–18.9)	12.8 (6.6–21.7)	0.1573
MuSK Ab detection rate (95% CI), %	1.2 (0.03–6.3)	5.8 (1.9–13.1)	0.0455
Sensitivity (95% CI)			
AChR RIA-positive cohort (N = 66), %	98.5 (91.9–99.9)	100 (94.6–100)	0.3173
MuSK RIA-positive cohort (N = 40), %	100 (91.2–100)	100 (91.2–100)	1.000
Specificity (N = 100 controls)			
AChR CBA (95% CI), %	100 (96.4–100)	100 (96.4–100)	1.000
MuSK CBA (95% CI), %	100 (96.4–100)	100 (96.4–100)	1.000
Interrater agreement (positive/negative result) (Cohen's kappa and 95% CI)			
All samples	0.978 (0.954–1.000) Agreement: 98.97%	0.972 (0.944–0.999) Agreement: 98.63%	—
Interrater agreement (CBA score) (95% CI)			
Adult AChR	0.925 (0.906–0.939)	0.876 (0.845–0.902)	—
Fetal AChR	0.832 (0.793–0.864)	0.890 (0.862–0.913)	—
MuSK	0.923 (0.904–0.939)	0.973 (0.965–0.979)	—
Interlaboratory agreement (positive/negative result) (Cohen's kappa and 95% CI)			
All samples (N = 96)	0.952 (0.885–1.000) Agreement: 97.92%	0.905 (0.814–0.996) Agreement: 95.83%	—

Abbreviations: Ab = antibody; AChR = acetylcholine receptor; CBA = cell-based assay; MuSK = muscle-specific tyrosine kinase; RIA = radioimmunoassay; RIA-dSN MG = radioimmunoassay double-seronegative myasthenia gravis.

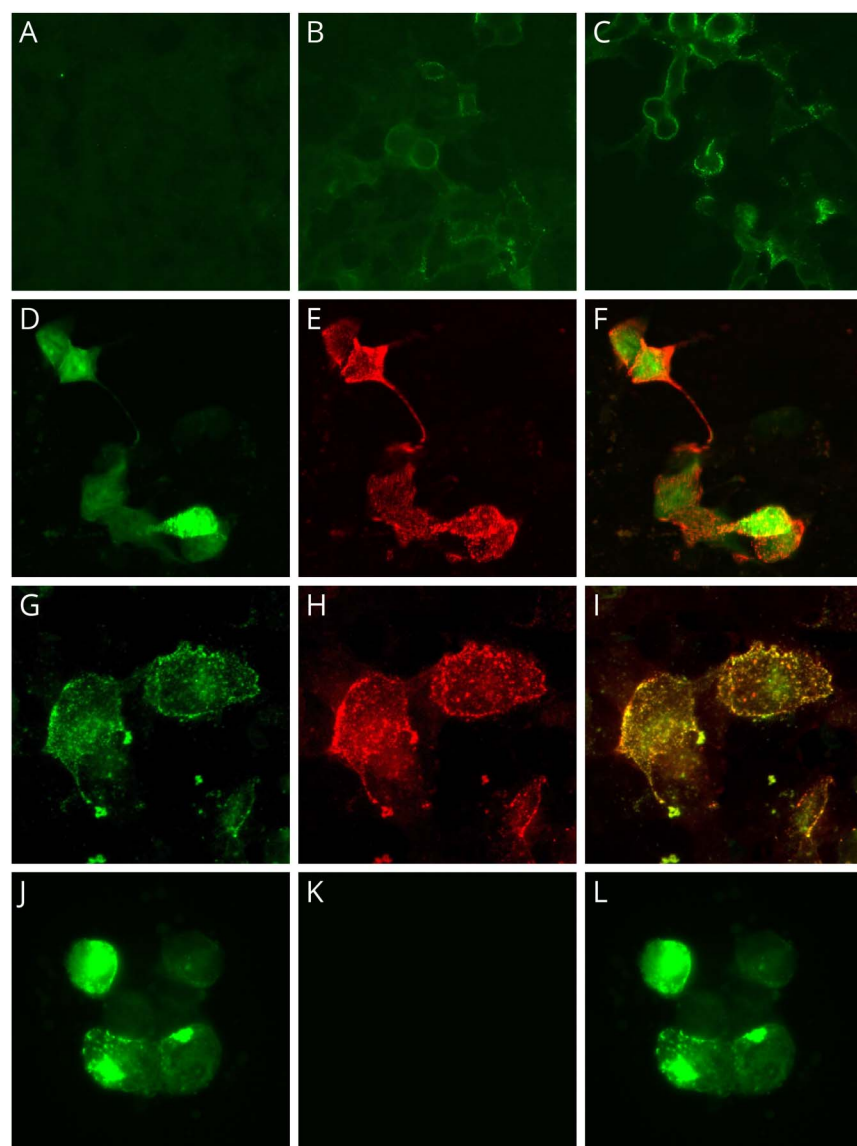
In the RIA-positive cohort, the sensitivity for AChR Abs was 98.5% for fixed CBA (95% CI 91.9–99.9), missing the detection of 1 low-positive sample and 100% for live CBA (95% CI 94.6–100) ($p = 0.1573$, eFigure 2, links.lww.com/NXI/A749). The sensitivity for MuSK Abs was 100% for both fixed and live CBAs (95% CI 91.2–100). Live and fixed CBAs for AChR and MuSK Abs resulted negative in all control sera (specificity = 100%, 95% CI 96.4–100; Figure 1, A, L–N).

Of interest, 2 samples were positive for only fetal AChR Abs. The first was from a RIA-negative female patient (SNMG-07 in eFigure 1, links.lww.com/NXI/A749), who had purely ocular MG with onset at the age of 42 years and treated with pyridostigmine and corticosteroids. At the last follow-up visit, 17 years after the disease onset, her post-intervention status (Myasthenia Gravis Foundation of America classification) was minimal manifestations. The second serum was from a RIA-positive male patient (AChR-20 in eFigure 1; AChR RIA titer 2.80 nMol/L), who was also affected by purely ocular MG with onset at the age of 65 years and treated only with pyridostigmine, with an post-intervention status (Myasthenia Gravis Foundation of America classification) of minimal manifestations at the last follow-up visit, 3 years after the disease onset.

The interrater agreement for positive/negative results was almost perfect for both fixed (Cohen's kappa = 0.978, 95% CI 0.954–1.000, agreement: 98.97%) and live CBAs (Cohen's kappa = 0.972, 95% CI 0.944–0.999, agreement: 98.63%). The interrater agreement for the CBA score ranged from good to excellent for both assays (ICC range: 0.832–0.973), with the lowest agreement observed for fetal AChR by fixed CBA and the highest for MuSK by live CBA. The interlaboratory agreement for positive/negative results was almost perfect for both fixed (Cohen's kappa = 0.952, 95% CI 0.885–1.000, agreement: 97.92%) and live CBAs (Cohen's kappa = 0.905, 95% CI 0.814–0.996, agreement: 95.83%).

Finally, to confirm the CBA results assessed by fluorescence microscopy, a proportion of samples were tested by FACS assay (representative plots are shown in eFigure 3, links.lww.com/NXI/A749). Among RIA-dSN sera, CBA positivity was confirmed by FACS analysis in all cases. All RIA-positive samples ($n = 81$, 53 AChR positive and 28 MuSK positive) were confirmed, and no false-positive results were found among the 67 controls tested (30 healthy subjects and 37 disease controls).

Figure 1 Fixed and Live Cell-Based Assay Result by Fluorescence Microscopy (Magnification: 40×)



Fixed CBA showing a negative result (A), a low-positive result for adult AChR Abs (B), and a positive result for MuSK Abs (C). (D-F): Live CBA for adult AChR Abs. (G-I): Live CBA for MuSK Abs. HEK293 cells transfected with AChR-rapsyn-eGFP (green), bound serum Abs from a patient with MG (red), and signal colocalization demonstrating Ab binding to transfected cells. Healthy control sample tested on eGFP-tagged live cells transfected with adult AChR (J) shows no binding (K), without signal colocalization (L). Ab = antibody; AChR = acetylcholine receptor; CBA = cell-based assay; eGFP = enhanced green fluorescent protein; HEK293 = human embryonic kidney cells 293; MuSK = muscle-specific tyrosine kinase.

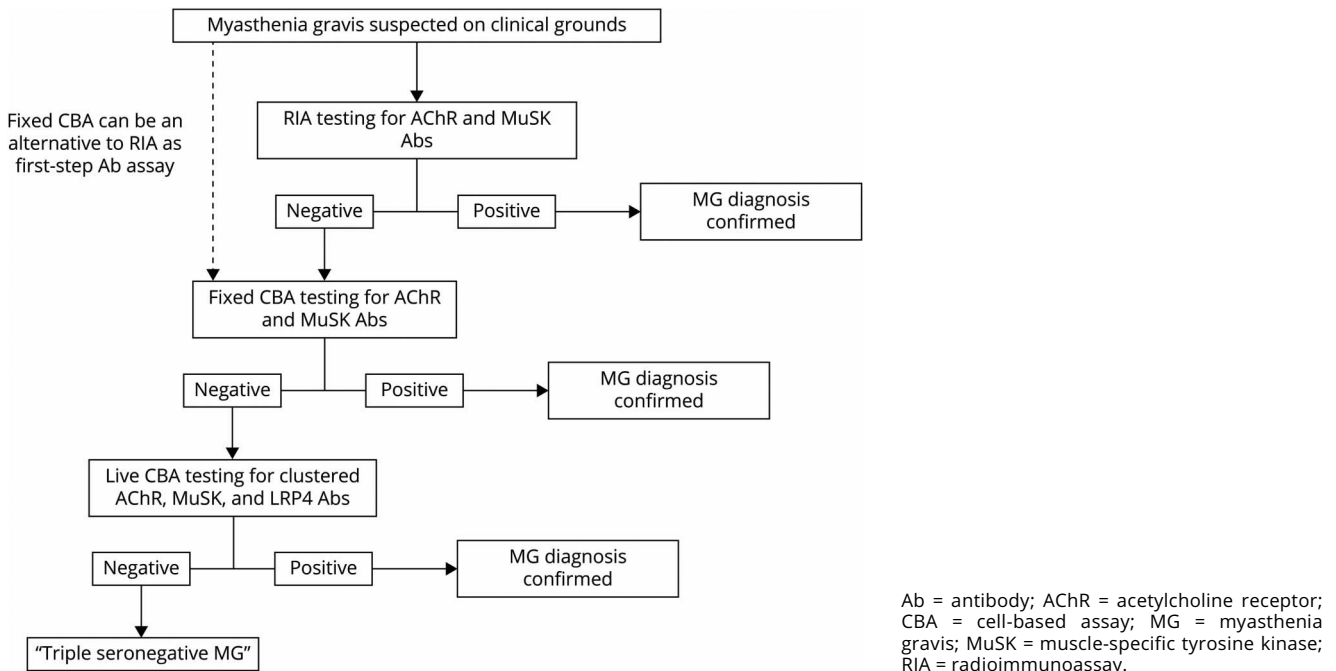
In summary, we confirmed that fixed CBA has an increased capability to detect AChR and MuSK Abs compared with RIA and can therefore be useful in the serologic evaluation of patients with RIA-dSN MG or as a first-step diagnostic test. An algorithm with a proposed testing sequencing for the serologic assessment of patients in whom MG is suspected on clinical grounds is shown in Figure 2.

Discussion

Our findings confirm that live and fixed CBAs both yield high sensitivity in RIA-positive patients as well as excellent specificity, in line with previous results.^{4,7,8,10} In RIA-dSN MG, live CBA allows the detection of clustered AChR and MuSK Abs in a proportion of patients.⁴⁻⁸ This is in line with previous reports, including a recent study from our group,⁸

and it reflects the use of a high-sensitive assay (live CBA) that can also detect low-affinity antibodies.⁹ A commercial fixed CBA for AChR and MuSK Abs has recently become available, and only a single study so far has assessed its performance, reporting a 4% increase in the AChR Ab positivity rate compared with RIA.¹⁰ Our comparative study found that these CBAs do not perform equally, with live CBA being more sensitive than fixed CBA ($p = 0.0143$) in the detection of both MuSK Abs and—to a lesser extent—AChR Abs. Indeed, 8% of RIA- and fixed CBA-negative samples were found to be positive only by live CBA. Notably, the difference in sensitivity between CBAs mostly pertained to MuSK Ab detection, with fixed CBA missing 4 of 5 samples positive by live CBA. According to these findings, negative samples by fixed CBAs should be referred to specialized centers for live CBA testing (algorithm for the serologic diagnosis of MG shown in Figure 2).

Figure 2 Algorithm Showing the Antibody Testing Sequencing When MG Is Suspected on Clinical Grounds



It is important that all RIA-dSN but CBA-positive patients had typical MG clinical features and treatment response, with an overall milder disease severity and higher frequency of purely ocular forms, as already described in patients with clustered AChR MG or with MuSK Abs solely detected by CBA.^{5,8} Notably, 2 patients, both with purely ocular MG, had Abs targeting only the fetal isoform of AChR, which highlights the importance of testing for these Abs to maximize the CBA sensitivity.

Our results are in line with previous studies that compared fixed and live CBAs for the detection of neural Abs, finding a reduced sensitivity of fixed CBA.¹²⁻¹⁴ Indeed, fixation could cause protein tertiary structure modifications, which may possibly mask relevant epitopes,^{14,15} thus preventing the binding of low-affinity Abs, particularly sensitive to such alterations.⁴ Fixed CBA has relevant practical advantages because it is less technically demanding and time consuming than live CBA and is affordable.¹⁶ Compared with RIA, an additional benefit is the lack of radioactive ligands. These advantages may result in a wide diffusion of this assay, with a likely improvement in MG diagnosis, favoring earlier immunotherapy and an overall better disease management.

Notably, both CBAs had excellent specificity, with no positive results in the control cohort, which included patients with other immune-mediated and neuromuscular disorders. However, testing of larger cohorts and a real-life evaluation of the assay performances are required to confirm these findings.

Despite providing a higher sensitivity than the standard RIA, CBAs—whether live or fixed—have also some drawbacks. First, the subjective assay interpretation can undermine its reproducibility.¹⁶ Nonetheless, in this study, live CBA assessed as positive by fluorescence microscopy was confirmed by FACS assay, and the interrater and interlaboratory agreements were excellent, supporting the result reliability and replicability, respectively. Second, CBAs assessed through fluorescence microscopy do not provide quantitative results, although this limitation can be overcome—to a certain extent—by Ab titration through progressive sample dilutions.¹⁶ Alternatively, live CBAs can be assessed through flow cytometry, which could represent a technical improvement providing a quantification of Ab binding.¹⁷ The FACS results shown here need to be confirmed in a larger cohort of patients and controls. Finally, it is relevant to point out that although Ab titers and their changes, as assessed by RIA, have been shown to correlate with the MG clinical course,^{18,19} the possible application of CBA to clinical monitoring of patients with MG during their follow-up has yet to be demonstrated.

In our study, the performance of fixed and live CBAs was compared in a large cohort of patients with MG from 2 centers. Based on our results, fixed CBA can be a valuable alternative to RIA and could be considered as a first-step diagnostic test, provided that the raters are experienced in result assessment. Furthermore, live CBA could be useful in the serologic evaluation of RIA- and fixed CBA-negative samples, particularly for patients harboring MuSK Abs, and the use of FACS represents a further improvement by providing quantitative results. Further studies on an independent, larger and unselected cohort of patients with MG and controls are needed to confirm these findings.

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Disclosure

The authors have nothing to disclose. Go to [Neurology.org/NN](https://www.neurology.org/NN) for full disclosures.

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