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Development of an Assay for Modulating Anti-Acetylcholine Receptor Autoantibodies Using Human Rhabdomyosarcoma Cell Line

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Three types of autoantibodies against the acetylcholine receptors (AChR) of skeletal muscle are detectable in patients with myasthenia gravis including binding, blocking, and modulating anti-AChR antibodies. Modulating autoantibodies correlate best with the severity of the disease, but are also technically most difficult to measure because the assay generally requires fresh human muscle cells. We have developed an assay for the modulation of anti-AChR antibodies using a rhabdomyosarcoma (RD) cell line expressing AChR on the cell surface. By decreasing the FetalClone III serum from 10% to 0.5% in Eagles Minimal Essential Medium (EMEM) we were able to increase the number of AChR on RD cells to meet the need of sensitivity of the assay. The extent of modulation was determined as the percent of AChR internalized in the presence or absence of modulating autoantibodies. Less than 6% modulation was found with the normal serum (n = 42). The CVs of both the intra- and day-to-day precision were less than 20%. When clinical samples (n = 105) were assayed in our laboratory and also at Nichols Institute, a correlation coefficient of 0.816 was obtained. The selection of RD cell line, the success of increasing the expression of the AChR on RD cells and the use of ^{125}I α-bungarotoxin of high specific activity allowed the establishment of an assay which can be used in routine clinical laboratory for the measurement of modulating anti-AChR autoantibodies for the management of patients with myasthenia gravis. J. Clin. Lab. Anal. 12:315–319, 1998.

Key words: myasthenia gravis; bungarotoxin; endocytosis; acetylcholine receptor

INTRODUCTION

Myasthenia gravis (MG) is an autoimmune disorder that affects neuromuscular function by impairing synaptic transmission. Three different types—including binding, blocking, and modulating—autoantibodies against muscle-type acetylcholine receptor (AChR) are detectable in patients with MG (1–3). The differences among these autoantibodies relate largely to where they bind on the surface of the receptor. Presence of these autoantibodies in MG patients may not only block the binding site of acetylcholine, but also lead to a decrease in the number of AChR at neuromuscular junctions by accelerating the rate of AChR internalization. As a result, the normal neuromuscular transmission mediated by acetylcholine become impaired causing muscle weakness and fatigue in patients (1,2,4).

Modulating antibodies are a group of autoantibodies that are responsible for the acceleration of AChR degradation at the muscle cell surface. These autoantibodies will bind to extracellularly exposed antigenic sites of two AChRs, triggering endocytosis and degradation. In studies performed by Drachman et al. (5) it was found that as many as 91% of MG patients have immunoglobulins which increase the rate of receptor degradation; moreover, the relative increase in the degradation rate corresponded closely to the severity of the disease of MG patients. Combining the effects of the antibody degradation and blockage of the AChR when monitored simultaneously, an index predicting the patients' clinical status was as high as 98%.

Because modulation of AChR is metabolism-dependent, viable human skeletal muscle cells must be used for the detection of modulating autoantibodies. Rat skeletal muscle cells have been used in the past, but are not the ideal substrate for routine clinical laboratory use. A human cell line, TE671, a medulloblastoma cell line established by McAllister et al. (6) was found to express muscle-like AChR (7–11). Although it is surprising for a neuronal cell line to express muscle-type receptors, the TE671 cell line proved useful not only in serv-

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ing as a reliable source for soluble AChR, but also in allowing the establishment of more specific and sensitive assays for blocking and modulating autoantibodies (2,5,8). However, medulloblastoma cell line TE671 is not readily available, especially for use in the clinical laboratory. It is stated in the American Tissue Culture Collection (ATCC) catalogue that "this material is cited in a US and/or other Patent and may not be used to infringe the patent claims."

Recently, it was reported that the TE671 cell line could be a subline of the rhabdomyosarcoma cell line (RD), a cell line from a malignant embryonal rhabdomyosarcoma of the pelvis of a 7-year-old Caucasian female (12). Several features of TE671 cells are shared with the RD cell line and are typical of muscle cells, such as the presence of desmin, myoglobin, and the muscle-type acetylcholine receptor. Cytogenetic studies also support that TE671 cells are a subline of RD cells (13). Since RD cells are commercially available and are commonly used in virology laboratories, we decided to investigate whether the RD cell line expresses AChR and can be used to establish an assay for measuring modulating anti-AChR autoantibodies (14).

Here we describe an assay to measure AChR modulating autoantibodies based on the accelerated internalization of the ^{125}I α-bungarotoxin-AChR complex using RD cells. The use of a 24-well microplate allows larger numbers of specimens to be assayed in the clinical laboratory.

MATERIALS AND METHODS

The RD cell line (CCL-136) was purchased from American Type Culture Collection (ATCC, Rockville, MD). ^{125}I α-bungarotoxin or ^{125}I α-butx {3-[^{125}I]iodotyrosyl}α-bungarotoxin) was purchased from Amersham (Arlington Heights, IL) at 2000 Ci/mmol. The 24-well flat bottom plates for the assay were purchased from Falcon (Franklin Lakes, NJ). Eagles Minimal Essential Medium (EMEM) powder was from GIBCO-BRL (Grand Island, NY). FetalClone III was purchased from HyClone (Fetal Clone III is a synthetic serum replacement for fetal bovine serum from HyClone Laboratories, Logan, UT), The BCA protein assay reagent (bicinchoninic acid) was from Pierce Chemical Co. (Rockford, IL). The majority of the chemicals used in this study including amino acids and vitamins were from Sigma Chemical Co. (St. Louis, MO).

Preparation of RD Cells

RD cells obtained at passages 33–45 were grown in EMEM in flasks (175 $cm^{2)}$ in a 37°C, 5% CO_2, 95% air humidified incubator. EMEM growth medium was supplemented with 2.2 g/L sodium bicarbonate, 1X MEM essential and nonessential amino acids, MEM vitamin solution, 50 U/mL penicillin, 50 μg/mL streptomycin, 2.5 μg/mL amphotericin B, 20 μg/mL gentamycin and 10% FetalClone III. Confluent monolayers of RD cells were detached using porcine pancreatic trypsin (2 mg/mL) supplemented with 0.005 M EDTA in calcium- and magnesium-free phosphate buffered saline. After a 2–3 min incubation, the cell suspension was removed and centrifuged at 1,500×*g* for 5 min. The pellet was resuspended in 10 mL EMEM and contained approximately 1.7 mg of cellular protein and 1.6×10^{-13} moles of AChR. AChR concentrations were determined following the procedure described previously (15).

Protein Determination

A microplate procedure for protein determination using the BCA reagent was used to measure protein concentration in the eluates. To each well, 10 μL of the sample was mixed with 0.2 mL of BCA working reagent and incubated for 45 min at room temperature. The plate was read at 562 nm on a microplate reader from Molecular Devices (Menlo Park, CA). BSA concentrations ranging from 0 to 2 mg/mL reconstituted in phosphate buffer saline (PBS) were used to construct a standard curve.

Assay for Modulating Anti-AChR Autoantibodies

1. Grow RD cells in EMEM growth media containing 10% FetalClone III serum for one week.
2. Plate the RD cells into 24-well plates at 6×10^4 cells/well in 2 mL of 10% EMEM.
3. Incubate the 24-well plates in a 5% CO_2, 95% humidified air incubator at 37°C for 4 days.
4. Decant the media then add 2 mL/well of EMEM growth media containing 0.5% FetalClone III. Incubate for an additional 2 days.
5. Wash each well twice with 1 mL of diluent media (MEM, 2.2 g/L sodium bicarbonate and 5.96 g/L HEPES).
6. Mix 2 μL of ^{125}I α-Butx with 1 mL of diluent media and add 0.25 mL of this mixture to each well. Incubate at room temperature for 3 hr on an orbital shaker.
7. Discard the supernatant and wash 3 times with 1 mL/well of diluent media; drain between each wash on adsorbent paper.
8. Prepare controls and patient samples in diluent medium (0.04 mL of the patient's serum in 1 mL of diluent media).
9. Add 0.4 mL of each control or patient sample to the appropriately labeled well. Each sample is assayed in duplicate.
10. Incubate plates in a 5% CO_2, 95% humidified air incubator at 37°C for 16–18 hr.
11. Remove supernatant and count radioactivity in a gamma counter.
12. Add 1 mL of 5 M NaOH to each well and rotate plates at room temperature for at least 30 min.
13. Remove cells and count cell associated radioactivity on gamma counter.

14. Calculate percent of modulation for each sample using the following formula:

$$\% \text{ Modulating} = \{1–[A/B]\} \times 100$$

A = sample pellet CPM/sample Total CPM
B = zero calibrator pellet CPM/zero calibrator Total CPM

Total CPM = CPM supernatant + CPM pellet

Calibrator and Control Preparation

Zero calibrator

Normal human sera from blood bank were pooled (HIV and hepatitis negative) and adjusted to 50% ammonium sulfate to remove immunoglobulins by precipitation. The supernatant, after dialysis against PBS was stored at –70°C and used as zero calibrator.

Negative control

Normal human sera from blood bank tested negative for modulating anti-AChR autoantibodies and for HIV and hepatitis were pooled and stored at –70°C for use as negative control.

Positive control

Patient samples tested positive for modulating anti-AChR autoantibodies were pooled and stored at –70°C.

Specimens

All specimens were obtained from Special Chemistry Laboratory at ARUP. They were sent to the Special Chemistry Laboratory for anti-AChR binding antibodies and from patients who were diagnosed clinically with myasthenia gravis.

RESULTS

Increase of AChR Expression

Development of a sensitive modulating assay using RD cells requires that there is a sufficiently high density of AChR on the cell surface and a high specific radioactivity of 125I α-Butx. Although the specific radioactivity of 125I α-butx purchased from Amersham at 2000 Ci/mmol meet our needs, however, the number of AChR present on the RD cells was not sufficiently high. To increase the AChR density on RD cells, various growth conditions for RD cells were investigated. As shown in Figure 1, when the concentration of FetalClone III was reduced from the normal 10% to 0.5%, a twofold increase of AChR was observed on second-day culture. Even though the cells had not reached confluency by the second day, the RD cells contained sufficient surface AChR to meet our needs of a sensitive assay. By combining the increased number of AChR and high specific 125I α-butx radioactivity, a sufficient amount of radioactivity was internalized and then released into the supernatant to allow detection of even small amounts of modulating autoantibodies.

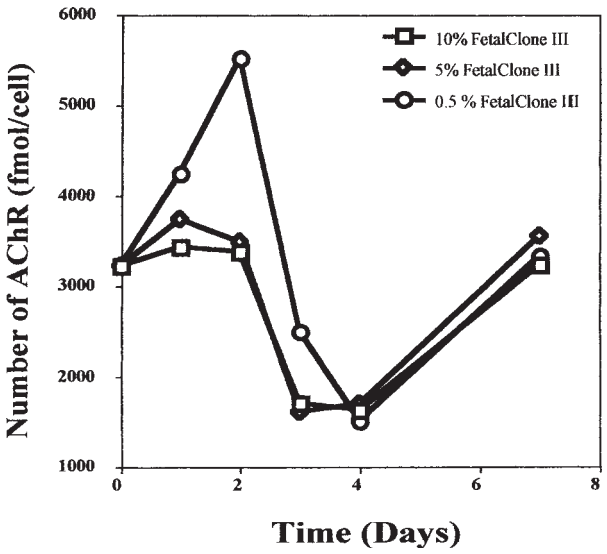

Fig. 1. Increase of the expression of AChR on RD cells by reducing the concentration of FetalClone III in the growth medium. Reducing the FetalClone III from 10% to 0.5% effectively increases the AChR density on RD cells. The highest AChR density was reached on the second day of culture.

Effect of FetalClone III

In order to maintain RD cells in a viable state during the assay we included FetalClone III in the assay mixture. For reasons still unclear to us, we experienced some difficulties in measuring the percent of modulation in the presence of FetalClone III. We could not find any difference in the modulation percentage of a zero calibrator and of a sample containing high modulating antibodies. At last we found out that it was the FetalClone III present in the assay mixture inhibiting the process of accelerated internalization. As shown in Figure 2, the percent of modulation decreased as the FetalClone III concentration increased in the assay buffer. The highest modulation percentage for both controls was observed when all FetalClone III was removed from the assay mixture. Fortunately the RD cells remained viable under FetalClone III-free conditions in the presence of human serum specimens. On the other hand, we felt it was probably a good idea to include 0.5% FetalClone III in our current assay mixture even though there was a slight inhibition of AChR modulation by the 0.5% FetalClone III. The presence of 0.5% FetalClone III did not seem to affect the larger difference in the percent of modulation between the low and high control but would keep the RD cell in a more viable metabolic state.

Sensitivity, Linearity, and Precision

A patient's serum containing highly elevated modulating autoantibodies was diluted serially in normal human serum and was used to determine assay linearity and sensitivity. As shown in Figure 3, our assay is linear between 47% and (ap-

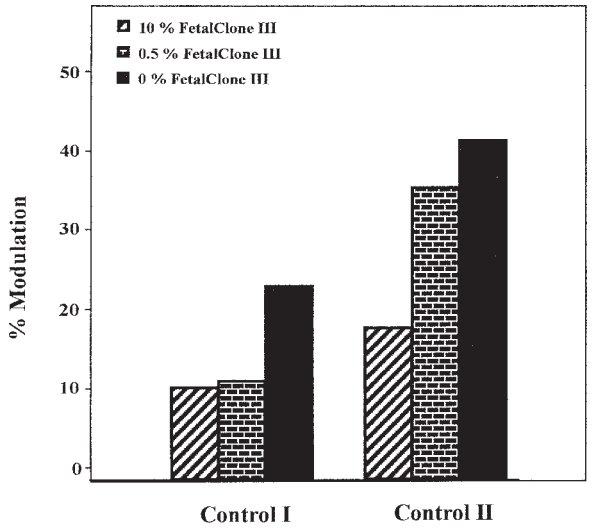

Fig. 2. Inhibition of autoantibody induced AChR modulation by FetalClone III. Two controls containing different levels of modulation autoantibodies were incubated with RD cells in the presence of various concentrations of FetalClone III. As the FetalClone III concentration was increased the percent of modulation was suppressed.

proximately) 15% modulation. The modulation percentage started to level off at further dilution of the sample. It appears from the dilution curve (Figure 3) that the sensitivity of our assay is approximately 5%. Therefore, our assay would not be able to distinguish percent of modulation caused by autoantibodies below that level. In addition, when the zero control was used to determine the analytical sensitivity of the assay, the mean ± SD was equal to 1.2 ± 1.7 (%). In other words, based on a 95% confidence level, the sensitivity can not be lower than 4.6 % modulation. Therefore, two different approaches came to the very similar conclusion that our assay sensitivity is about 5% modulation.

TABLE 1. Precision of Modulating Anti-AChR Autoantibodies Assay

Within-day precision			
N	Mean (% modulation)	SD	CV (%)
11	19	3	16
11	31	3	6
Day-to-day precision			
Mean (% modulation)	SD	% CV	N
30	5	17	9
48	5	10	9

The CVs of both the intra- and day-to-day precision were listed in Table 1 and they are both less than 20%.

Comparison With Nichols Institute

Samples were divided into two equal sets. One set was assayed for the modulating autoantibodies by our in-house assay and the second set was sent to Nichols Institute. A comparison between our determinations and results from Nichols Institute is shown in Figure 4. Despite a great deal of scattering between the assay values, the correlation coefficient was very good (g = 0.816). Considering the procedure of this bioassay and the difference in the two assay designs, we felt that our assay is comparable to that of Nichols Institute. Except for a few samples, Nichols Institute's results

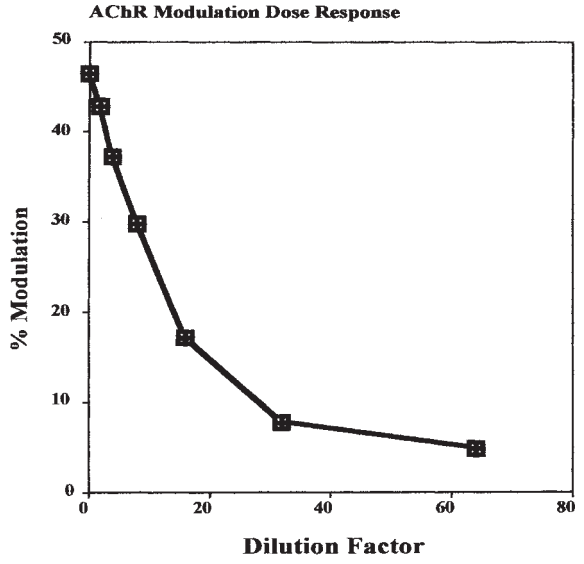

Fig. 3. Assay sensitivity and concentration range. A sample containing highly elevated modulating antibodies was serially diluted and subjected to our in-house assay. It appears that our assay is linear down to 15% and the sensitivity of the assay is close to 5% modulation.

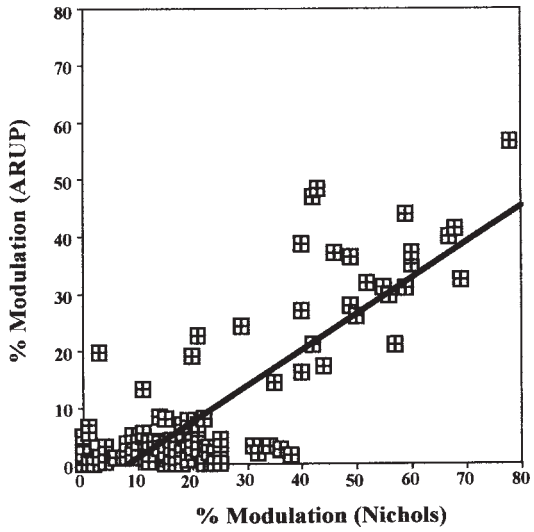

Fig. 4. Correlation of modulation percentage between the results of our in-house assay and the assay results made by Nichols Institute on the same specimens (n = 105).

tended to be slightly higher than ours. Whether this was due to a difference in sensitivity or specificity is not known.

Normal Range

To establish the normal reference values for modulating autoantibody we have assayed 42 normal specimens from the blood bank with no known neuromuscular or autoimmune diseases. The mean ± SD of the percent of modulation antibodies was 1.8 ± 1.9. Since our assay sensitivity was 5%, the results suggested that almost no modulating autoantibodies were present in these specimens. Based on a 95% confidence level (±2 SD), we adopted an upper normal cutoff at 5.6 % modulation.

Stability

Modulating autoantibodies appear very stable. Both controls stored at either 4°C or –70°C for one month did not show any significant loss in percent of modulation (Figure 5).

DISCUSSION

Of the three types of anti-AChR autoantibodies (binding, blocking, or modulating) the modulating antibody assay is the most difficult test to perform. Cells that remain metabolically active and express high levels of AChR are required for the assay development. For this reason, fresh human muscle biopsies or human muscle cultures have been used to measure receptor internalization (16). Conceivably, our discovery of the RD cell made it possible to establish the assay for routine clinical laboratories without having to use human muscle. On the other hand, our success of inducing a high density of AChR on the RD cell surface and the use of 125I α-butx from Amersham of high specific radioactivity also made possible for us to develop an assay with sufficient sensitivity for managing MG patients. It should be noted that glucocorticoid and dexamethasone have also been shown to significantly increase the total surface AChR on cultured human muscle cells (8,16). Whether or not this approach has any advantage over the procedure we are using needs to be further investigated.

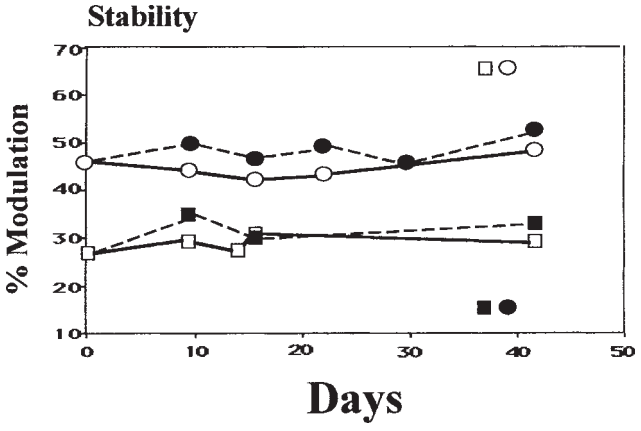

Fig. 5. Stability of modulating autoantibody at two concentration levels, stored at 4°C (open symbols) and –70°C (solid symbols).

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