



# Serum Protein Electrophoretic Pattern in Experimental Myasthenia Gravis

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**Abstract:** Myasthenia gravis (MG) is an antibody-mediated autoimmune neuromuscular disease, leading to skeletal muscle weakness. The autoantibodies are against acetylcholine receptor (AChR) of the muscle membrane localized at the neuromuscular junction. The early diagnosis of MG is a key factor for the advanced medical practice, but, it still remains challenging. The objective of this pilot study was to examine the pattern of serum protein electrophoresis in the animal experimental model of MG. Acetylcholine receptor (AChR) was purified from rat (mail Wistar) leg muscle by affinity chromatography. Four rabbits were immunized on day 1, week 4 and week 8 with purified rat leg muscle AChR and assayed for serum anti-AChR antibody titer on blood samples taken on week 2, week 5 and week 9. Control rabbits received an emulsion of phosphate buffered saline in the adjuvant. The serum anti-AChR antibodies were tittered using quenching fluoroimmunoassay. Electrophoresis separation of the serum proteins was performed on a cellulose acetate membrane. Results showed that Immunizations of rabbits induced muscle weakness in the animals together with elevation of serum anti-AChR antibody. During the course of immunizations, percentage of beta-globulin fraction increased gradually from 15.8% to 41.2% whereas, albumin decreased from 68.3% to 43.8%. As determined by cellulose acetate electrophoresis. These results represent proof-of-principle data for diagnosis of the acetylcholine receptor-MG subtypes and severity.

**Keywords:** Acetylcholine, Autoimmune Disease, Electrophoresis, Fluoroimmunoassay, Myasthenia Gravis

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## 1. Introduction

Myasthenia gravis (MG) is a chronic autoimmune neuromuscular disease characterized by varying degrees of skeletal muscles weakness. A major part of the pathological process is known to be associated with the impairment of neuromuscular transmission. In most cases skeletal muscle acetylcholine receptor (AChR), is reported to be the main target of the autoimmune attack [1-4]. This in turn results in fewer AChR available for stimulation, which leads to less muscle fiber activation, and the ultimate development of muscles weakness. Because muscles weakness is a common symptom of many other disorders, the diagnosis of MG is often missed in people who experience mild weakness or in those individuals whose weakness is restricted to only a few muscles [5, 6]. At present, diagnostic investigations of MG

include tension test, electromyography and serum antibodies titer estimations. Each of these measures, however, has its own set of limitations. The risk of side effects of intravenous injection of edrophonium chloride (an inhibitor of acetylcholinesterase), in tension test and the danger of causing bradycardia and apnea is reported to be very subjective [7-9]. Single fiber electromyography (EMG) measures the electrical potential of muscle cells when single muscle fibers are stimulated by electrical impulses [10, 11]. EMG examination is particularly useful in uncooperative patients or when it is desirable to control the firing rate precisely, or during voluntary muscle activation, but may not be specific to a particular muscles weakness disease. The studies on the measurement of antibody indicated that although elevated amounts of antibodies specific for AChR were detected in about 85 percent of the patients, the titer of the antibodies in different patients corresponds poorly with

severity of the disease. A similar result has also reported in experimental animal model of MG [14, 15]. Nevertheless, there are no circulating serum biomarkers that correlate with the disease state between patients or different MG subtypes. It may fulfill the need of a simple alternative approach in order to confirm the type and or possibly treatment response in MG. Furthermore, it seems to be of interest whether certain serum proteins might account in part for their ability to produce different degrees of clinical severity of the disease. In this paper, we report the finding of our study on the relationship of the pattern of serum protein electrophoresis, antibody titers and clinical responses in rabbits immunized with rat muscle AChR.

## 2. Experimental Methods

### 2.1. Materials

Naja Naja alpha - bungarotoxin was obtained from Razi institute, Hesarak, Iran. Fluorescein isothiocyanate, phenylmethyl sulfonyl fluoride (PMSF), sucrose, Tris-HCl, acrylamide, bis-acrylamide, Triton X-100, 2-mercaptoethanolamine, SDS, ammonium persulfate, EDTA, EGTA, Sepharose-4B, Sephadex G-25, fine grade, acetylcholine-chloride, bovine serum albumin (BSA), Freund's adjuvant (complete and incomplete), cellulose acetate membrane, pore size 0.8  $\mu$ m, diam. 47 mm were purchased from Sigma (Poole, Dorset, UK). Unless stated otherwise, all reagents were of the highest grade and made up in double glass-distilled water.

## 3. Methods

### 3.1. Purification of Leg AChR

Male Wistar rats (200-250 g) were killed by decapitation and leg muscles were dissected on ice and chopped into the consistency of a mince. The blood and other debris were washed off and the chopped tissue was then homogenized in 5 volumes of isolation medium (10 mM Tris buffer pH 7.4 containing; 12 mM NaCl, 1 mM EDTA, 1 mM PMSF and 0.5% Triton X-100) in a Virtis 45 homogenizer. This homogenate was diluted to 60ml with isolation medium and centrifuged at 1000 g for 10 min at 4°C. The pellet was discarded and the supernatant was centrifuged at 27000 g for 20 min. The pellet was resuspended in the cold isolation media containing 1% Triton X-100 and centrifuged at 27000 g for 20 min. at 4°C. The supernatant was used for further purification of AChR by affinity chromatography, on a conjugate of Naja Naja alpha - bungarotoxin to Sepharose-4B, and the competitive elution with acetylcholine [16]. The eluted AChR was dialyzed against water before use. The determination of the protein concentration was performed by Lowry's method [17].

### 3.2. Immunization

Four rabbits (Albino 1.5-1.7 kg) were anesthetized by

injection of 1 ml of 50 mg/kg sodium pentobarbital i. p. When the animals were asleep, they were injected with the purified AChR emulsified in Freund's complete adjuvant as schedule recommended by Landon and Moffat [18]. The animals were immunized on day 1, week 4 and week 8 and assayed for serum anti-AChR antibody titer on blood samples taken on week 2, week 5 and week 9. Control rabbits (n=4) received an emulsion of phosphate buffered saline in the adjuvant. The animals were kept warm on a heating pad to prevent hypothermia for 2 h.

### 3.3. Fluorescein-Labeled AChR

Fluorescein-labeled AChR was prepared as described by Messripour and Moein [19]. Equal volumes of FITC solution (1 mg/mL) and purified AChR solution (about 0.25 mg protein/mL) were mixed and stirred overnight at 4°C. The labeled F-AChR was separated from unconjugated FITC using Sephadex G-25 columns (1.2  $\times$  20 cm).

### 3.4. Determination of Anti-AChR Antibodies

The antibodies raised against rat muscle AChR were titered by indirect quenching fluoroimmunoassay essentially as described by Messripour and Moein. Briefly, fluorescein-labeled AChR (100  $\mu$ l) was added to the tubes containing 100  $\mu$ l of the rabbit serum sample. After 5 min, 100  $\mu$ l anti-fluorescein serum (1:100) was added, and incubation at room temperature for 15 min. The volume was then increased up to 2 ml by addition of the buffer and fluorescence intensities of the mixture were measured using a Perkin-Elmer (Norwalk, CT) LSE spectrophotofluorometer with the excitation wavelength set at 495 nm and emission of wavelength at 540 nm. In all experiments, a correction was made for the background signal contributed by reactions other than that of the fluorescein-labeled-AChR. Rabbit anti-fluorescein dilution curve was used to measure the titer anti-AChR antibodies in the immunized rabbits. All samples were run in duplicate, and the average value is reported.

### 3.5. Cellulose Acetate Electrophoresis

The electrophoresis separation of the rabbit serum protein fractions was performed on a cellulose acetate membrane. After an application of 2.5  $\mu$ l of serum on a 10-cm cellulose acetate strip, the samples were subjected to electrophoresis in barbital buffer, pH 8.6 and ionic strength 0.075, for 30 min at 160 v with a mean current of 2.5 mA per strip. After the proteins are separated, the membrane is placed in a solution of Trichloroacetic acid and Ponceau S (to stain the protein bands). Destaining solution, permits the appreciation of the 5 protein fractions: albumin, alpha1, alpha2, beta, and gamma proteins.

### 3.6. Statistical Analysis

Statistical analysis was performed using the Statistical Package of Social Science (SPSS) version 17. Data are presented as means  $\pm$  standard deviation (SD). Experimental data from the values obtained from immunized animals are

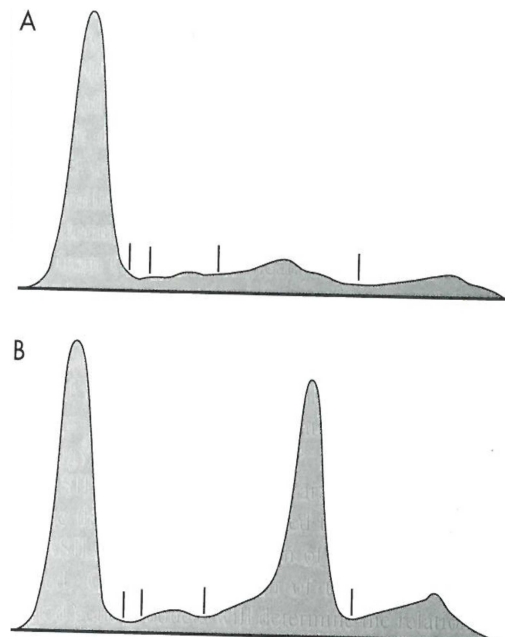
compared to their own values taken before immunizations by pairing Student's t-test. The level of significance was set at  $P < 0.005$ .

## 4. Results and Discussions

### 4.1. Results

After multiple immunizations with rat AChR, rabbits developed muscle weakness, flaccid paralysis and decreased activity. The fatigue head down was coincided with bending of the front limbs of the animals. The pathogenesis aspects were observed in the animals as early as 2 weeks post-injection and were developed to severe generalized weakness as late as week 9. This observation is consistent with previous reports on rat immunizing with *Torpedo californica* AChR [20]. During the period of muscle weakness blood samples were taken and tested for the anti-AChR antibody titer and acetate cellulose electrophoresis. Table 1 shows anti-AChR antibody titer of the 4 rabbits during the course of immunization with purified rat muscle AChR. At week 2 post-primary immunizations the antibody titer of rabbit 1 which showed muscle weakness was similar to that of rabbit 4 which showed normal muscle activities. A marked increase in the anti-AChR antibody titer was seen 1 week after the last boosting at week 9. Fractionation of proteins by electrophoresis was conducted on the same serum samples. As shown in Figure 1 beta-globulin fractions in the immunized animals increased significantly at week 9 as compared to that of control values ( $P < 0.005$ ), though the

enhancements were observable from week 2. The levels of albumin, however, decreased at week 5 and continued done at week 9. The pooled data from 4 rabbits are summarized in Table 2.



**Figure 1.** Electrophoretic pattern of serum samples taken from a control rabbit (A) and an immunized rabbit with rat leg muscle AChR (B).

**Table 1.** Time course of anti-AChR antibody titer in individual rabbits immunized with rat leg muscle AChR.

Rabbit No	Before immunization	Week 2 post immunization	Week 5 after boosting	Week 9 after boosting
1	1:20	1:40	1:200	1:800
2	1:40	1:100	1:200	1:1600
3	1:20	1:200	1:400	1:3200
4	1:20	1:400	1:400	1:800

Four rabbits were immunized on day 1, week 4 and week 8 with purified rat leg muscle AChR and assayed for serum anti-AChR antibody titer on blood samples taken on week 2, week 5 and week 9. Control rabbits received an emulsion of phosphate buffered saline in the adjuvant. Anti-AChR antibody titer was measured by an indirect fluororeceptor assay.

**Table 2.** Changes of the rabbit serum protein fractions during the course of immunization with rat leg muscle AChR.

Protein fractions	Before mmunization (%)	Week 2 post mmunization (%)	Week 5 after boosting (%)	Week 9 after boosting (%)
Albumin	68.3±2.1	63.4±4.3	49.7±3.3*	43.8±38
Alpha1 globulin	1.3±0.1	1.4±0.1	1.4±0.2	0.8±0.0
Alpha 2 globulin	5.5±0.7	4.9±0.2	4.1±0.3	3.6±2
Beta globulins	15.8±0.8	20.6±0.9	36.0±1.3*	41.2±1.5*
Gama globulin	9.1±0.4	9.7±0.6	8.8±0.6	10.6±0.9

Four rabbits were immunized (see legend o Tulle1) and serum protein fractions were separated electrophoresis on a cellulose acetate membrane. The results are expressed as mean percentage  $\pm$  SD of 4 serum samples.  $P < 0.005$  compared with the values of the serum samples taken before immunizations.

### 4.2. Discussions

The present study assessed for the first time the alterations

of serum protein fractions in rabbit experimental autoimmune myasthenia gravis. The repeated immunizations of rabbits induced muscle weakness in the animals together with elevation of anti-AChR antibody in the circulation, which is one of the best characteristic changes reported in autoimmune MG [1-4]. The existence of a close relationship between the development of muscle weakness and the percentage of beta-globulin fractions in the serum of individual immunized rabbits, is in accord with the

electrophoresis pattern reported on the patients with autoimmune disease of acute disseminated encephalomyelitis [21]. However, The results is inconsistent with just one previous on the serum electrophoresis pattern of MG patients that believed to be hypergammaglobulinaemia [22]. Considering the electrophoresis patterns of this report, it seems that high levels of beta covered gamma globulin. This might occurred partly due to the weak separation of the proteins; thus misinterpreting the results. Under normal conditions beta-globulins are synthesized and shed by many cells, particularly lymphocytes, and is detectable in the circulation of normal individuals. The beta globulin fraction also contains proteins that act as carriers of substances in the blood. These proteins are transferrin, beta-2 macroglobulin, cytokines and the complement component C3. Functions for these proteins as the modulator of lymphocyte and as a potential regulator of the immune system are revealed [23, 24].

## 5. Conclusion and Recommendations

### 5.1. Conclusion

It is concluded therefore that, the purified rat muscle AChE antigen might trigger up-regulation of some components of beta-globulins during the course of immunizations. This suggestion can be defended by up-regulated profile of inflammatory proteins in sera of MG [25]. In addition a number of membrane molecules, including ligand-gated ion channel and Ion channel receptors, such as nicotinic ACh receptors and NMDA receptors [26, 27]. These channels and other membrane proteins at the neuromuscular junction may be targeted by autoantibodies leading to abnormal neuromuscular transmission.

### 5.2. Recommendations

It is recommended that although nonspecific, serum protein electrophoresis might be consider as a diagnostic implement of MG severity. However, there is a lack of evidence that, the need for human MG population is still relevant limitations to its use.

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