

Research Article

Comparative Analysis of the Informative Value of Radioimmunoassay and Laser Correlation Spectroscopy in Myasthenia Gravis

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Received 19 January 2014; Accepted 19 February 2014; Published 12 March 2014

Academic Editors: M. Bakhiet and A. A. Manfredi

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The objective of this study was to compare informative value of traditional approach (anti-AChR antibody radioimmunoassay) and evaluation of metabolic shifts by laser correlation spectroscopy in myasthenia gravis. The method based on changes in spectral characteristics of laser radiation caused by scattering in a disperse system yields a histogram reflecting particle contribution into light scatter as a function of particle radius in nanometers. The spectrum of anti-AChR-positive serum is characterized by appreciably increased contribution of particles with a radius 4.6–6.2 nm. Binding of serum components with solubilized AChR confirms that this peak is determined by elevated concentration of antibodies to this receptor. The search for the relationship between the disease severity and the distribution pattern of subfraction serum components revealed three informative zones: 6–15, 27–67, and 127–223 nm. In patients without disturbances of vital functions, the contribution of the first zone particles into light scatter increases and that of the third zone particles decreases. Considerable differences attaining the level of statistical significance in zones 4–6 and 20 nm were revealed in the spectra of serum from patients with myasthenia gravis of the same severity with and without thymoma. This opens prospects for dynamic monitoring of the efficiency of therapy.

1. Introduction

According to current concept of myasthenia gravis (MG) pathogenesis, autoimmune aggression towards certain molecular structures of the skeletal muscle and neuromuscular junction is the leading pathophysiological factor responsible for the development of clinical manifestations of this disease [1–3].

Numerous studies have demonstrated that postsynaptic nicotinic acetylcholine receptors (AChR) are more often attacked in MG: 80–85% myasthenia patients have elevated concentrations of antibodies to these receptors [4].

Pathophysiological role of antibodies to AChR has been confirmed in animal experiments reproducing the development of myasthenic process after immunization with AChR preparations or injection of the serum from myasthenic

patients [5, 6]. Antibody-mediated autoimmune processes impair neuromuscular transmission due to loss of functionally active AChR, which impairs acetylcholine binding to the receptor and reduces the probability of the transmitter-receptor interaction. Moreover, antibody fixation to the postsynaptic membrane leads to destruction of junction folds and structural modification of the synapse. Hence, not only the density of AChR decreases, but also the configuration of the synaptic cleft is altered [7].

The presence of high concentration of anti-AChR antibodies in the majority of patients with generalized MG prompted using this parameter as an important diagnostic laboratory marker. At the same time, the correlations between the levels of these antibodies and clinical parameters, in particular, disease severity, were analyzed. Some authors believed that the severity of clinical manifestation in some patients

correlates with the serum level of anti-AChR antibodies. For instance, Krarup in 1984 reported significant correlation between the titer of anti-AChR antibodies and MG severity [8]. Evoli with coworkers in 1996 also demonstrated this correlation, the strongest correlation between the disease severity and the titer of antibodies to AChR being observed in patients with thymoma [9].

However, later studies disproved this assumption. The absence of correlations between serum concentrations of anti-AChR autoantibodies and the severity of clinical symptoms as well as patients' age, sex, and duration of the disease was demonstrated not once.

Thus, elevated concentration of antibodies to AChR is a reliable diagnostic marker, but due to the absence of correlations with other clinically important parameters, the use of this parameter is confined to the diagnostic tests [10].

Apart from laboratory tests, integral diagnostic methods are rapidly developed. These approaches go far beyond simple data accumulation and imply evaluation of mutual influences and combinations of various parameters and their correlations with clinical symptoms and other indexes. One of these methods, laser correlation spectroscopy (LCS), that is, analysis of native biological fluids, is successfully used in clinical practice. The method is based on measurement of the spectral characteristics of quasielastic light scattering (Rayleigh scattering) in the studied system by the spectrum of intensity fluctuations of the recorded light. The recorded intensity fluctuation spectra of the scattered light are mathematically processed by the regularization method [11] using a special procedure included in the spectrometer software. This mathematical apparatus for the solution of inverse spectral problem is similar to that used successfully in modern X-ray and NMR tomography. The LCS method was previously applied for evaluation of the disease severity and efficiency of treatment in patients with asthma [12], diabetes mellitus [13, 14], myasthenia [15], and other pathologies.

We have previously demonstrated [15] that the contribution of immunoglobulin fraction into light scattering increased in patients with mild MG, while in patients with severe disease, autoimmune components predominated. However, low number of examined individuals suffering from this disabling disease prompted us to continue our studies. Moreover, it was interesting to compare the data of traditional radioimmunoassay with the results of LCS analysis.

The objective of this study was to compare informative value of traditional approach (anti-AChR antibody radioimmunoassay) and evaluation of metabolic shifts by LCS in MG.

2. Materials and Methods of the Study

A total of 77 patients with MG at the age of 12–80 years (mean 41.7 ± 17.5 ; $M \pm \delta$) were examined; the group included 23 men (29.9%) at the age from 17 to 80 years (51.6 ± 16.4) and 54 women (70.1%) at the age from 12 to 76 years (37.5 ± 16.1). Thymoma was diagnosed in 8 patients (10.4%).

The distribution of patients by the severity of clinical manifestation of MG according to MGFA criteria [16] revealed heterogeneity of the examined group by this parameter: isolated weakness of ocular muscles (1) was detected in

4 patients (5.2%), 2 A in 17 patients (22.1%), 2 B in 16 patients (20.7%), 3 A in 12 patients (15.6%), 3 B in 24 patients (31.2%), and 4 B in 4 patients (5.2%).

MG was diagnosed on the basis of clinical examination and electromyography results and the data of pharmacological tests with proserine (neostigmine) and Kalymin-Forte (pyridostigmine bromide). Thymoma was diagnosed on the basis of CT or MRI of the anterior mediastinum.

The severity of clinical manifestation of MG was assessed by a 5-point scale according to international clinical classification (4.5), where 1 corresponded to weakness of ocular muscles without involvement of other muscles and 2A-4B corresponded to increasing weakness of the body and bulbar muscles (A and B indicate the absence and presence of bulbar disorders, resp.).

The concentration of antibodies to acetylcholine receptors (AChR) was measured by radioimmunoassay using a commercial test system (DLD Diagnostica GMBH, Germany). The serum samples were stored at -20°C . For the analysis, they were defrosted; $5 \mu\text{L}$ aliquots were transferred to tubes, mixed with $100 \mu\text{L}$ ^{125}I -AChR (specific activity 342 Ci/mmol) and $50 \mu\text{L}$ antibodies to human IgG, and incubated for 30 min at room temperature. Then, 1 mL washing buffer was added, the mixture was centrifuged at 3000 g for 20 min, the supernatant was decanted, and the pellet was resuspended and washed again. Sample radioactivity was measured for 1 min on a radioactivity counter (Clinigamma, LKB, Sweden) and the concentration of antibodies (nmol/liter) was calculated considering the test system manufacturing date, specific radioactivity of the label, sample radioactivity, and radioactivity of negative control:

$$C = (\text{cpm}_i - \text{cpm}_n) \times D \times F, \quad (1)$$

where C is antibody concentration, nmol/liter, D is the correction factor for test system manufacturing date (e.g., 1.98), F is the correction factor for specific radioactivity of the label (e.g., 0.38×10^{-3}), cpm_i is the sample radioactivity, cpm , and cpm_n is the negative control radioactivity, cpm .

The concentration of anti-AChR antibodies $>0.40 \text{ nmol/liter}$ was considered elevated.

Subfraction composition of biological fluids was recorded on a laser correlation spectrometer (LCS-03-INTOKS) approved by Committee for New Medical Equipment, Ministry of Health of the Russian Federation, for measuring microparticle size in biological fluids (Certificate RU.C. 39.003.A N 5381).

The method is based on changes in spectral characteristics of monochromatic coherent He-Ne laser radiation caused by scattering in a disperse system (plasma, serum, urine). The sample (0.2 mL) was placed in a cuvette of an LCS spectrometer. Measurements were performed at a frequency of 16 kHz (2000 accumulations). Spectrum regularization was performed using a nonlinear scale (Spectrometer and Blood software supplied with the spectrometer). This algorithm yields a histogram reflecting particle contribution into light scatter (ordinate) as a function of particle radius in nanometers (abscissa).

The LCS data were presented in different variants. Detailed LC histograms formed by the spectrometer operational program consisted of 32 columns. The number of columns corresponds to the number of molecular subfraction analyzed during spectrum processing (minimization) [11, 17]. Less detailed, but more convenient variant of data presentation implies discretization into zones comprising particles with a certain radius. For blood serum, the entire spectrum can be divided into 5 zones: (1) 0–10 nm; (2) 11–30 nm; (3) 31–70 nm; (4) 71–150 nm; (5) >150 nm. According to a priori information obtained using various models of pathologies, the first interval (0–10 nm) primarily includes low-molecular-weight monomer albumins and free glycolipid complexes; the second interval (11–30 nm) includes globular proteins and low-molecular-weight lipoprotein complexes; the third interval (31–70 nm) contains larger lipoprotein complexes, RNP and DNP particles, and immune complexes with the lowest molecular weight; the fourth interval (71–150 nm) includes constitutive medium-molecular-weight immune complexes; induction of immunopoiesis with the formation of high-molecular-weight immune complexes (usually associated with allergization and autoimmune sensitization) leads to the appearance of larger particles (>150 nm).

3. Results and Discussion

For evaluation of the size and localization of anti-AChR antibodies on LC histogram, two experiments were performed:

- (a) comparison of the histograms of anti-AChR-negative and anti-AChR-positive sera;
- (b) binding analysis of components of anti-AChR-positive serum with solubilized AChR from cultured human cells (DLD Diagnostica GMBH, Germany).

As seen from Figure 1, LC histograms of the two sera differ significantly. The spectrum of anti-AChR-positive serum is characterized by appreciably increased contribution of particles with a radius 4.6–6.2 nm, that is, close to the immunoglobulin zone.

Binding of serum components with solubilized AChR confirms that this peak is determined by elevated concentration of antibodies to this receptor (Figure 2). Figure 2 shows that 2 h serum incubation with the receptor considerably modified the particle distribution and induced the appearance of two zones with enhanced light scatter corresponding to particles with radiuses of 8–20 and 166–300 nm. The first zone was probably associated with the appearance of simple antigen-antibody complexes, while the other was determined by large immune complexes formed under conditions of receptor excess. Serum incubation with physiological saline induced no changes in subfraction composition.

As expected, no correlations were found between the content of anti-AChR antibodies and the contribution of the corresponding fraction into light scatter, because all immunoglobulins have similar size and the presence of other antibodies in the studied zone contributing into light scatter is possible.

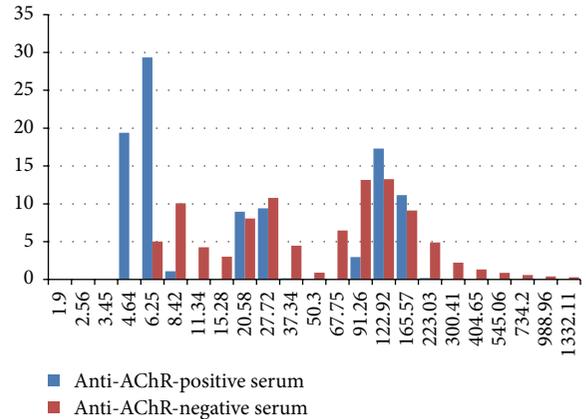


FIGURE 1: Particle size distribution histograms in anti-AChR-negative and anti-AChR-positive (3.9 nmol/liter) sera. Abscissa: particle radius, nm. Ordinate: contribution into light scatter, %.

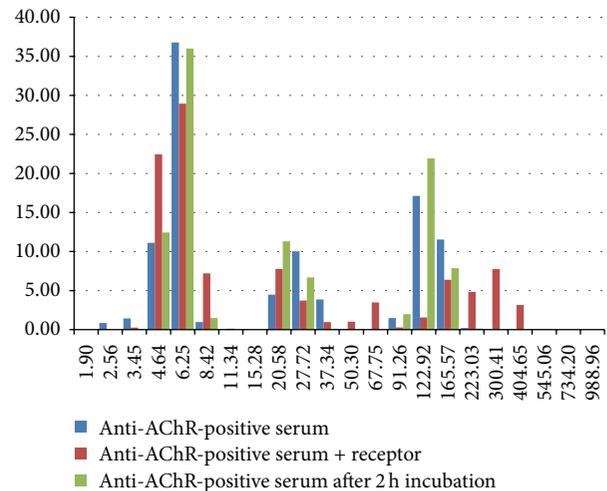


FIGURE 2: Size distribution histograms of serum particles and products of its interaction with solubilized AChR. Abscissa: particle radius, nm. Ordinate: contribution into light scatter, %.

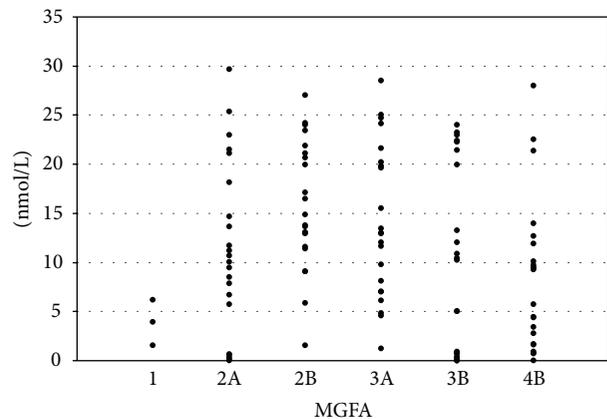


FIGURE 3: Concentration of anti-AChR antibodies in patients with myasthenia gravis of different severity. Abscissa: disease severity. Ordinate: antibody concentration, nmol/liter.

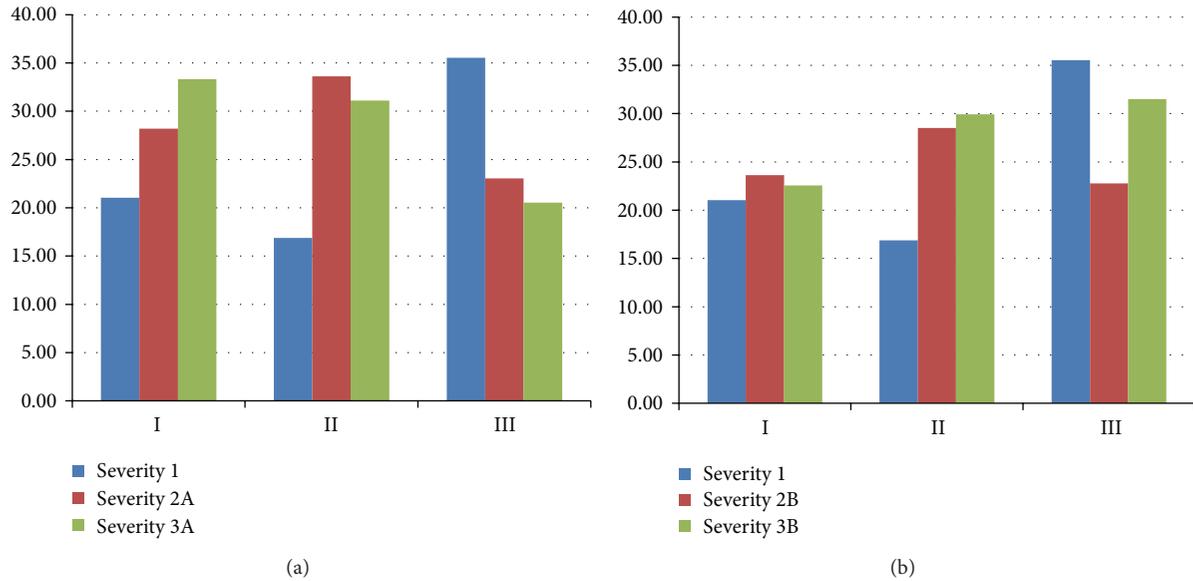


FIGURE 4: Averaged contribution of serum particles into light scatter in blood serum samples from patients ($n = 74$) with myasthenia gravis of different severity without (a) and with vital function impairment (b). Abscissa: informative zones I, II, and III (6–15, 27–67, and 127–223 nm, resp.). Ordinate: contribution into light scatter, %.

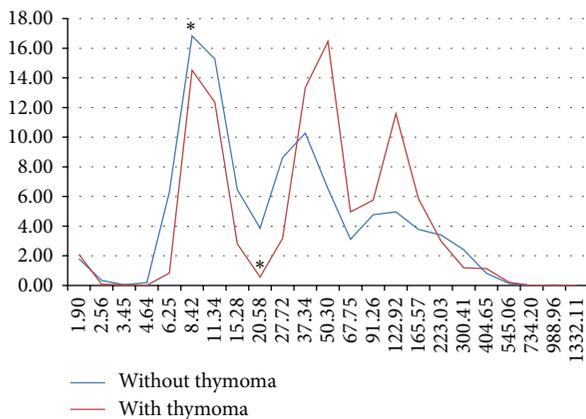


FIGURE 5: Serum particle size distribution in patients with myasthenia gravis of the same severity with thymoma ($n = 6$) and without tumor ($n = 6$). Abscissa: particle radius, nm. Ordinate: contribution into light scatter, %. *significant differences by the Mann-Whitney U test, $P < 0.05$.

The search for the relationship between the disease severity and the distribution pattern of subfraction serum components was of particular interest, because there is no correlation between the concentration of anti-AChR antibodies and activity of the pathological process in MG. The latter fact was confirmed in our study (Figure 3): the content of anti-AChR antibodies was similar in patients with grade 1, grade 2, and grade 3 MG. At the same time, our previous results brought hope for detection of specific features of spectra using the LCS method [10].

Analysis of histograms revealed three informative zones including 75–84% of total light scatter: 6–15, 27–67, and

127–223 nm. Figure 4 shows averaged contributions of these zones into light scatter in patients with MG of different severity. As is seen in Figure 4(a), in patients without disturbances of vital functions, the contribution of the first zone particles into light scatter increases and that of the third zone particles decreases. This opens prospects for dynamic monitoring of the efficiency of therapy in the examined group. In patients with impaired vital functions (Figure 4(b)), the picture drastically differs, which reflects essential changes in body functioning and confirms informative value of the used approach. Because of low number of patients with grade 4 MG, their parameters are not presented in figures, but we observed the appearance of very large (300–400 nm) particles, which agreed with the previous reports.

Considerable differences attaining the level of statistical significance in zones 4–6 and 20 nm were revealed in the spectra of blood serum from patients with MG of the same severity with and without thymoma (Figure 5). Predominance of large particles in patients with thymoma is apparently determined by proliferative processes in the body.

Elevated titer of anti-AChR antibodies along with clinical manifestations of the disease, positive response to anticholinesterase preparations, and electromyographic phenomena reflecting disturbances in neuromuscular transmission are classical diagnostic criteria of autoimmune MG. At the same time, in some patients (~20%), the titer of anti-AChR antibodies does not differ from that in healthy individuals (seronegative MG) [18]. Anti-AChR antibodies are usually undetectable in patients with Lambert-Eaton and congenital myasthenic syndromes [19, 20]. On the other hand, elevated concentrations of anti-AChR antibodies were found in cirrhosis of the liver, Hashimoto syndrome, and rheumatoid arthritis [21]. These facts explain both the increased concentrations of anti-AChR antibodies in the

majority of the examined patients and the absence of correlation between the concentration of antibodies and disease severity. It appears that the standard set of clinical, electrophysiological, and pharmacological tests for myasthenia and myasthenic syndromes should be supplemented with an analysis capable of evaluating biochemical correlates of the disease severity.

Our findings suggest that LCS allows evaluation of the severity of the pathological process. The observed increase in the content of immunoglobulin zone components probably plays a regulatory role. In clinical practice, immunoglobulins in high doses suppressing the immune processes are used for the treatment of MG as the alternative for plasmapheresis. It can be hypothesized that the sharp increase in the content of immunoglobulin fraction in patients with mild MG is a regulatory reaction aimed at suppression of autoantibody production. At the same time, reduced contribution of this fraction observed in patients with severe course of MG with simultaneous increase in the content of immune complexes attests to insufficiency of the regulatory mechanisms and, hence, disease progression. Our findings suggest that LCS can supplement the modern armory of diagnostic methods used for evaluation of the disease severity and efficiency of therapy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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