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Case report

Predictive value of serum anti-C1q antibody levels in experimental autoimmune myasthenia gravis

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Abstract

Components of the complement cascade and circulating immune complexes play important roles in both experimental autoimmune myasthenia gravis and myasthenia gravis in humans. Thus far, no serological factor has been identified to predict the clinical severity of either myasthenia gravis. Upon immunization with acetylcholine receptor, levels of complement factors C1q, C3 and CIC increase with time in sera from C57BL/6 (B6) mice. Both these and plasma samples from myasthenia gravis patients also contain anti-C1q antibodies. The serum levels of anti-C1q antibodies but not C1q, C3 and CIC are significantly correlated with the clinical severity in the experimental myasthenia mice. However, this correlation is not observed in myasthenia gravis patients.

Keywords: Myasthenia gravis; Anti-C1q antibody; Circulating immune complexes; Experimental autoimmune myasthenia gravis; Autoimmunity

1. Introduction

Experimental autoimmune myasthenia gravis (EAMG) is a T cell-dependent, antibody-mediated autoimmune disease of the neuromuscular junction (NMJ), which closely resembles human myasthenia gravis (MG) in its clinical and pathological manifestations [1]. Extensive research performed on both MG and EAMG in recent decades has identified various clinical and immunological parameters associated with the natural course and pathogenesis of the disease. However, there is still no identified immunological factor that reliably predicts the presence or the clinical severity of MG. Even the serum levels of anti-acetylcholine receptor (AChR) antibodies, which are known to play a very crucial role in the development of EAMG and MG, are not correlated with the severity of clinical disease. Therefore, predictions on the outcome, the treatment schedule and the clinical course of MG are generally based on the patients' clinical signs and symptoms and thus on the clinical skills and experiences of individual physicians.

Recently, we have highlighted the significance of the classical complement pathway (CP) and circulating immune complexes (CIC) in the induction and the clinical severity of EAMG [1,2]. Our results demonstrated that anti-AChR antibodies produced in response to AChR-immunization have pathogenic effects only in the presence of the components of the classical and lytic pathways in EAMG [2,3]. Additionally, we also showed that serum CIC levels are correlated with the clinical severity of EAMG in RIIIS/J mice [1].

To assess the significance of anti-C1q antibody, another CIC-associated factor observed in some autoimmune diseases, we measured CIC-related factors in sera of mice with EAMG. Here, we show for the first time that these contain anti-C1q antibodies, which correlate with the muscle weakness of the mice. We also demonstrate that anti-C1q antibody is present in sera of MG patients.

2. Materials and methods

2.1. Patients

Plasma samples of 11 patients (one male, 10 female, ages between 26 and 61) with autoimmune, generalized MG

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were used in the study. The diagnosis was based on signs and symptoms of facial, ocular, bulbar, or extremity weakness with fluctuating severity and improvement with rest. All patients were positive for anti-AChR antibodies. Duration of MG ranged between 1 and 37 years and their quantitative MG scores varied between 9 and 21. All patients were steroid-dependent. Nine out of 11 patients had thymectomies (all more than 1 year prior to study entry). Seven out of these nine patients had thymic hyperplasia, whereas two patients had an atrophic thymus. None of the patients had thymomas.

2.2. AChR, E. coli proteins and mice

AChR was purified from the electric organs of *Torpedo californica* by a neurotoxin affinity column [1]. *E. coli* BL21 (DE3) cells (Novagen) were used for extraction of *E. coli* proteins. The cells were grown at 37 °C in LB medium containing streptomycin (50 μg/ml). Cells were harvested and centrifuged at $8000 \times g$ for 15 min. The cell pellet was washed in PBS at pH 7.3. Cells were mixed with 6 M urea containing phenylmethylsulfonylfluoride (1 mM), sonicated (10 s × 3) and incubated in a shaking water bath at 4 °C for 24 h. The presence of proteins was shown on SDS-PAGE performed in a Bio-Rad Mini-Protean gel. Seven- to 8-week-old C57BL/6 (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animals were housed in the viral Ab-free barrier facility at the University of Texas Medical Branch and maintained according to the Institutional Animal Care and Use Committee Guidelines.

2.3. Induction and clinical evaluation of EAMG

For in vivo studies, all mice were anesthetized and immunized with 20 μg of AChR emulsified in CFA (Difco, Detroit, MI) s.c. at four sites (two hind footpads and shoulders) on day 0. All of the mice were boosted with 20 μg of AChR in CFA s.c. at four sites on the back on day 30 (second immunization). For clinical examination, mice were left for 3 min on a flat platform and were observed for signs of EAMG. Clinical muscle weakness was graded as follows, grade 0, mouse with normal posture, muscle strength, and mobility; grade 1, normal at rest but with muscle weakness characteristically shown by a hunchback posture, restricted mobility, and difficulty in raising the head after exercise, consisting of 20–30 paw grips on cage top grid; grade 2, mouse showed grade 1 symptoms without exercise during observation period on flat platform; grade 3, dehydrated and moribund with grade 2 weakness; and grade 4, dead [1]. Eight B6 mice were immunized and boosted with 50 μg of *E. coli* proteins in CFA using the same protocol. These mice did not develop clinical disease following immunization.

2.4. Measurement of grip muscle strength

For objective measurement of muscle strength, mice were first exercised with 40 paw grips on cage top grid. Following exercise, mice were made to grasp a grid attached to a dynamometer (Chatillon Digital Force Gauge, DFIS 2, Columbus Instruments, Columbus, OH). While pulling them by their tails, we recorded the force required to make them loose their grip. For all statistical comparisons, the grip strength values obtained on day 45 after first AChR immunization were used. The grip strengths were assessed blind of serum anti-AChR antibody status and vice versa.

2.5. ELISA for anti-AChR antibody levels

Affinity-purified mouse AChR (0.5 μg/ml) was coated onto a 96-well microtiter plate (Dynatech Immulon 2; Dynatech Laboratories, Chantilly, VA) with 0.1 M carbonate bicarbonate buffer (pH 9.6) overnight at 4 °C. The plates were blocked with 2% BSA in PBS at room temperature for 30 min. Serum samples were diluted 1/1000 in PBS/0.05% Tween 20 and incubated at 37 °C for 90 min. After four washes, HRP-conjugated goat anti-mouse IgG (Caltag Laboratories, San Francisco, CA), diluted 1/1000 in PBS/0.05% Tween 20, was added and incubated at 37 °C for 90 min. Subsequently, ABTS (indicator) solution in 0.1 M citric buffer, pH 4.3, in the presence of H_2O_2 was added, and color was allowed to develop at room temperature in the dark. Absorbance values were measured at a wavelength of 405 nm, using a Molecular Devices (Sunnyvale, CA) Emax microplate reader, and the results were expressed as OD values.

2.6. ELISA for serum complement and circulating immune complex (CIC) levels

Serum C1q, C3, C1q-containing CIC (C1q-CIC) and C3-containing CIC (C3-CIC) levels were measured by ELISA. Briefly, 96-well microtiter plates (Dynatech Laboratories) were coated with goat antibody to mouse C3 (ICN Biomedicals/Cappel, Aurora, OH) or with an anti-C1q mAb (obtained and purified from hybridoma cells [4A4B11, ATCC, Manassas, VA] and shown to react with mouse C1q in our lab) overnight at 4 °C. The plates were then blocked with 2% BSA in PBS at room temperature for 30 min. Diluted (1/10 in PBS-0.05% Tween 20) serum samples were added and incubated at 37 °C for 90 min for C3 and overnight at 4 °C for C1q. After four washes, HRP-conjugated goat anti-mouse C3 (ICN Biomedicals/Cappel) or biotin-conjugated goat anti-mouse C1q (Cedarlane Labs, Hornby, Ontario) complement components diluted 1/500 in PBS/0.05% Tween 20, was added and incubated at 37 °C for 90 min for C3 and at room temperature for 45 min for C1q. For C3, ABTS substrate