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C5a is not involved in experimental autoimmune myasthenia gravis pathogenesis

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Abstract

C5 deficient mice are highly resistant to experimental autoimmune myasthenia gravis (EAMG) despite intact immune response to acethylcholine receptor (AChR), validating the pivotal role played by membrane attack complex (MAC, C5b-9) in neuromuscular junction destruction. To distinguish the significance of C5a from that of C5b in EAMG pathogenesis, C5a receptor (C5aR) knockout (KO) and wild-type (WT) mice were immunized with AChR to induce pathogenic anti-AChR antibodies. In contrast with C5 deficient mice, C5aR KO mice were equally susceptible to EAMG as WT mice and exhibited comparable antibody and lymphocyte proliferation response to AChR implicating that C5a is not involved in EAMG development.

Keywords: Myasthenia gravis; C5a; C5a receptor; Autoimmunity

1. Introduction

Myasthenia gravis (MG) and experimental autoimmune myasthenia gravis (EAMG) are both classical antibody-mediated diseases, characterized with muscle weakness and triggered by antibodies to acetylcholine receptor (AChR) located at the neuromuscular junction (NMJ) (Christadoss and Dauphinee, 1986; Vincent and Drachman, 2002). Recent years have brought growing awareness of the involvement of the complement system in anti-AChR mediated NMJ destruction in MG and EAMG (Tuzun et al., 2003; Tsujihata et al., 1989). In EAMG, activation of the complement cascade occurs by the classical pathway leading to C3 activation and generation of C3b. Subsequent cleavage of C5 leads to release of C5a and generation of C5b. C5b initiates formation of C5b-9 complex or membrane attack complex (MAC), which is involved in lysis of the postsynaptic NMJ membrane (Tuzun et al., 2003; Tsujihata et al., 1989).

Evidence for the participation of MAC (and therefore C5b) in EAMG pathogenesis comes from multiple sources. MAC has been observed in the postmortem frozen muscle specimens of EAMG mice (Tuzun et al., 2003) and the number of MAC deposits has been reduced in EAMG resistant mice (Tuzun et al., 2003; Tuzun et al., 2006). Further, administration of an antibody against the C6 component of MAC before the passive transfer of anti-AChR antibody has prevented EAMG induction in rats and has inhibited accumulation of MAC components C6 and C9 (Biesecker and Gomez, 1989). Also, C5 deficient mice immunized with AChR have been shown to be highly resistant to EAMG despite preserved anti-AChR antibody production ability (Christadoss, 1988).

So far, there has been no investigation on the possible participation of C5a in EAMG pathogenesis. It is not clear, for instance, whether EAMG resistance of C5 deficient mice (which are deficient for both C5a and C5b components) is solely due to impaired MAC production caused by the absence of C5b or also to the additional deficiency of C5a associated immune functions. C5a is a major anaphylactic and chemotactic agent and promotes production of cytokines (e.g. IL-1β, IL-6, IL-12) that are actively involved in EAMG pathogenesis (Morgan et al., 1992; O'Barr

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and Cooper, 2000). Therefore, inborn deficiency of C5a signaling through C5a receptor (C5aR) might be expected to influence EAMG susceptibility. Hence, to address that question, in this study we examined whether C5a receptor (C5aR) deficient mice were susceptible to MG utilizing an experimental model, EAMG.

2. Materials and methods

2.1. AChR and mice

AChR was purified from the electric organ of *Torpedo californica* by α-neurotoxin affinity column (Wu et al., 1997). Seven- to eight-wk-old C5aR KO mice in the B6 background were kindly provided by Dr. Girardi (Hospital for Special Surgery, NY, NY) and were generated by targeted deletion of the murine C5aR gene. C5aR KO mice were produced by backcrossing to B6 mice for six generations. The mice were determined to be completely C5aR deficient by PCR, Northern Blot and immunohistochemistry analysis. C5aR deficient mice were backcrossed with B6 mice as described in detail (Wenderfer et al., 2005; Girardi et al., 2003). Control wild-type (WT) B6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine, USA). All animals were housed in the viral antibody-free barrier facility at the University of Texas Medical Branch and maintained according to the Institutional Animal Care and Use Committee Guidelines.

2.2. Induction and clinical evaluation of EAMG

All mice were anesthetized and immunized with 20 μg AChR emulsified in CFA (Difco, Detroit, MI) s.c. at four sites (two hind footpads and shoulders) on day 0 and were boosted with 20 μg AChR in CFA s.c. at four sites on the back on days 30 and 60. 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, with muscle weakness characteristically shown by a hunchback posture, restricted mobility, and difficulty to raise the head after exercise, consisting of 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. 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). The maximal force applied to the dynamometer while pulling the mouse by its tail until it lost its grip on the grid was recorded. Clinical EAMG was also confirmed by i.p. administration of 50 μl neostigmine bromide, along with atropine sulfate in PBS, and observing improvement in muscle strength.

2.3. RIA to measure muscle AChR content

The total concentration of AChR per mouse carcass was determined according to a previously published method (Wu et al., 1997). Aliquots (0.1 ml) of [^{125}I] α-bungarotoxin (BTX)-labeled (5×10^{-9} M), Triton X-100 solubilized mouse muscle extracts, with and without benzoquinonium (10^{-3} M) and were mixed with 10 μl of mouse anti-AChR serum. The resulting complex was precipitated by goat anti-mouse serum and then centrifuged. Radioactivity of the pellet was counted in a Packard gamma counter (Packard Instrument Co., Meriden, CT), and cpm values of samples with benzoquinonium were subtracted from cpm values of samples without benzoquinonium. The results were expressed as picomolar (pM) of [^{125}I]-labeled BTX-binding sites per gram of mouse carcass.

2.4. ELISA for anti-muscle AChR antibody and isotypes

IgG, IgG1 and IgG2b isotypes to mouse muscle AChR were evaluated by ELISA, using a previously described method (Tuzun et al., 2003). These isotypes were examined due to their established significance in EAMG pathogenesis (Yang et al., 2005). Affinity-purified mouse AChR (0.5 μg/ml) was coated onto a 96-well microtiter plate in 0.1 M carbonate bicarbonate buffer overnight at 4 °C. Diluted serum samples of 100 μl (1:500) were added and incubated at 37 °C for 90 min. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG, IgG1 or IgG2b (Caltag Laboratories, Burlingame, CA) (1:1000) were added and then incubated at 37 °C for 90 min. Subsequently, the peroxidase indicator substrate 2,2′-azino-bis-(3-ethylbenzothiazoline 6-sulfonate) substrate (ABTS) solution in 0.1 M citric buffer (pH 4.35) was added in the presence of H_2O_2, and mixture was allowed to develop color at room temperature in the dark. Plates were read at a wavelength of

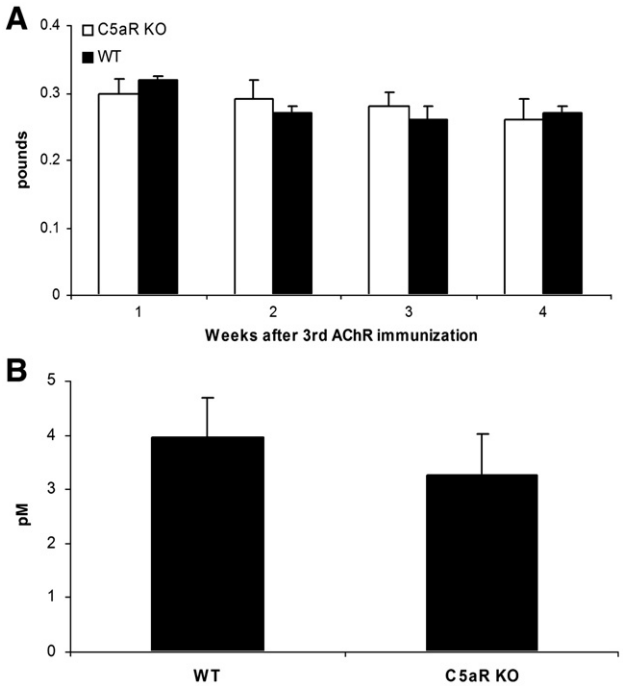

Fig. 1. Grip strength kinetics (A) and muscle AChR content (B) of AChR-immunized WT and C5aR KO mice following 3rd AChR immunization. C5aR KO mice were equally susceptible to EAMG as WT mice ($p>0.05$ by Student's *t*-test). Bars indicate standard errors.