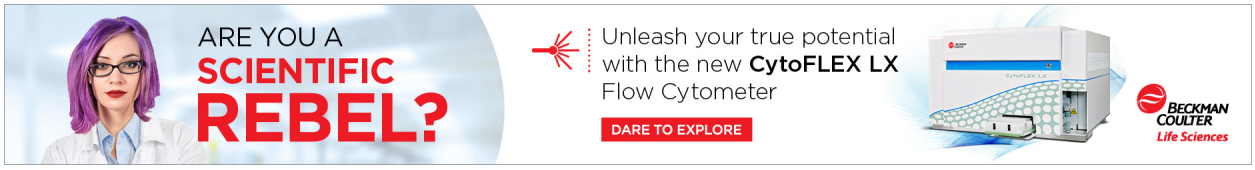

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J Immunol 2007; 179:8562-8567; ;
doi: 10.4049/jimmunol.179.12.8562
http://www.jimmunol.org/content/179/12/8562

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Print ISSN: 0022-1767 Online ISSN: 1550-6606.

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Anti-C5 Antibody Treatment Ameliorates Weakness in Experimentally Acquired Myasthenia Gravis[1]

Yuefang Zhou,* Bendi Gong,* Feng Lin,† Russell P. Rother,‡ M. Edward Medof,[2†] and Henry J. Kaminski[2,3*]

Myasthenia gravis (MG) is a neuromuscular transmission disorder in which damage to acetylcholine receptors (AChR) on motor endplates by autoantibody-induced complement attack causes muscle weakness. To determine whether and, if so, to what extent, blockade of complement cascade at the C5 step ameliorates disease, we evaluated the effect of administering a functionally blocking anti-C5 mAb in passive experimental MG in Lewis rats induced with AChR Ab McAb-3. In contrast to uniform severe weakness at 24 h requiring euthanasia in untreated animals, anti-C5 mAb-pretreated rats showed no weakness at 48 h. Anti-C5 mAb treatment 24 h after disease induction restored strength in two-thirds of the rats. Immunofluorescence staining of endplates from the treated animals showed that C9 deposition at AChR was reduced and ultrastructural analyses showed that endplates were intact. The results argue that targeting C5 may warrant testing in MG patients and that this approach may be particularly valuable for myasthenic crisis. ***The Journal of Immunology,*** **2007, 179: 8562–8567.**

In myasthenia gravis (MG),[4] patients produce nicotinic acetylcholine receptor (AChR) autoantibodies that cause neuromuscular transmission failure, eventuating in muscle weakness. Although life expectancy is not decreased (primarily due to improved management of acute respiratory muscle weakness ("myasthenic crisis")), more specific and more effective therapy is needed. Disease-related morbidity is high and both nonimmunosuppressive and immunosuppressive agents currently in use are variably effective with poor side effect profiles (1, 2).

In MG and experimental MG (EAMG), AChR Abs appear to act by directly blocking AChR function, accelerating AChR degradation, or through activation of complement. The deleterious effects of AChR Abs mediated through activation of complement (3–6) is supported by the observations that 1) C3 activation fragments as well as C9, and other components of the membrane attack complex (MAC), are uniformly detectable at motor endplates both in patients and animals with EAMG (7–9); 2) depletion of C3 by cobra venom factor protects rats against induction of EAMG (10, 11); 3) C6-deficient rats resist passive EAMG (12); and 4) in the active form of EAMG induced by AChR immunization, C5-deficient mice develop less severe disease (13). In further support of these data, studies by ourselves (14, 15) and others (16) have shown that mice deficient in intrinsic complement regulatory proteins DAF and CD59, which protect self-tissues from autologous complement injury, develop markedly more severe passively induced EAMG (14–16). Findings that C4-deficient mice are completely resistant to disease implicate the classical complement pathway in initiating complement activation (17). Taken together, these data strongly support complement activation and, in particular, assembly of the MAC at the motor endplate is the principal process underlying destruction of postsynaptic structure.

Consequently, targeting complement activation is a rational approach for MG disease management. Mounting evidence supports the feasibility of complement blockade as a therapeutic approach in MG (16, 18, 19). Administration of anti-C6 Ab (18) or soluble C3b receptor (CR1 or CD35), a C3/C5 convertase inhibitor (19), has been shown to protect rats against EAMG. In one study (16), administration of a mouse anti-mouse C5 mAb protected CD59-deficient mice from passive EAMG in the absence of CD59, the intrinsic regulator that protects self-cells against endogenous C5b-mediated injury.

Recent clinical studies have been performed with functionally blocking anti-human C5 Abs. Anti-C5 mAb (eculizumab) and a single chain version of the anti-C5 mAb (pexelizumab) have exhibited short-term safety in several human disorders including acute myocardial infarction (20) and coronary artery bypass graft surgery (21). The above anti-C5 mAb has demonstrated long-term safety and efficacy in paroxysmal nocturnal hemoglobinuria (23). In the present investigation, we tested an anti-rat C5 mAb for the ability to limit weakness in passive EAMG rats.

Materials and Methods

Passive induction of EAMG and treatments with anti-C5 mAb

Female Lewis rats weighing 150–180 g (Harlan Breeders) received i.p. injections of rat anti-mouse muscle AChR monoclonal IgG2b isotype Ab McAb-3 (a gift from V. Lennon, Mayo Clinic, Rochester, MN), which binds the skeletal muscle AChR, to induce EAMG. Preliminary experiments identified a 0.24-μg McAb-3/g rat produced mild weakness (class 1, see scale below) in 24 h and severe weakness in 48 h (classes 3–4). For therapeutic evaluations, a series of investigations was performed. In the first experiment, rats was injected with McAb-3 at time 0 while another group received simultaneous i.p. injections of McAb-3 and 5 mg (1.4 mg/ml) of functionally blocking mouse anti-rat C5. The anti-C5 mAb, designated 18A, is an IgG2b that binds the α-chain of rat C5 and is known to

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Received for publication June 2, 2007. Accepted for publication October 2, 2007.

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[1] This work was supported by National Institutes of Health Grants R24EY014837 and R01EY013238 (to H.J.K.) and Grant P30 EY11370.

[2] M.E.M. and H.J.K. contributed equally to this work.

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[4] Abbreviations used in this paper: MG, myasthenia gravis; AChR, acetylcholine receptor; EAMG, experimental MG; MAC, membrane attack complex.

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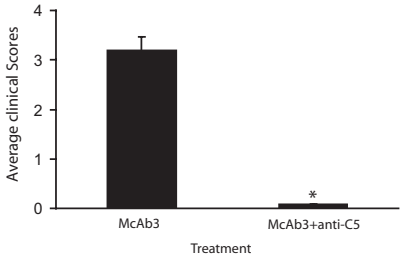

FIGURE 1. Strength scores of EAMG rats treated with or without anti-C5 mAb simultaneous with EAMG induction. Nine rats were injected i.p. with McAb-3 on day 0 and five of these rats received anti-C5 mAb simultaneously. Clinical scores were measured individually at days 1 and 2 and shown are clinical scores averaged from the same treatment group at day 2. Rats that did not receive anti-C5 mAb treatment demonstrated varying degrees of weakness and rats with clinical scores of 3 and greater at day 2 underwent euthanasia. Rats treated with anti-C5 mAb had a clinical score of 0 at both time points ($p < 0.000004$). Results are shown as means ± SE.

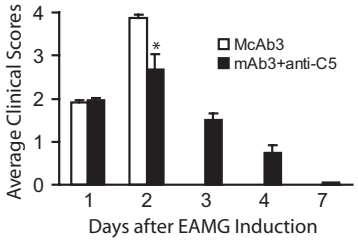

FIGURE 2. Clinical scores of rats treated with or without anti-C5 mAb 24 h after EAMG induction. On day 0, 11 rats were administered McAb-3 to induce EAMG. On day 1, these rats developed symptoms of EAMG and the clinical scores are around 2. Six of these rats were randomly chosen and treated with anti-C5 mAb. Although rats treated with anti-C5 mAb became slightly worse on day 2 compared with day 1, they had significantly better disease severity scores compared with untreated rats (*, $p < 0.014$) and the untreated rats developed such severe weakness that they underwent euthanasia. The anti-C5 mAb-treated rats that were not sacrificed for tissue collection gradually recovered. Results are shown as means ± SE.

block C5b-9-mediated hemolysis and C5a-dependent neutrophil migration (24). In the next experiment, rats were injected with McAb-3 and 24 h later with 5 mg of 1.4 mg/ml anti-C5 Ab. The third experiment involved three sets of rats 1) McAb-3 alone, 2) McAb-3 and anti-C5, and 3) McAb-3 and isotype control mAb 16C. The 16C control mAb (control IgG) binds to rat C5 but does not block C5b-9-mediated hemolysis or C5a-dependent neutrophil migration (24).

Assessment of muscle weakness

Rats were monitored for development of weakness, which was assessed by an accepted motor strength scale as follows: 0 = can grip and lift lid of a cage, 1 = can grip but cannot lift the lid of a cage, 2 = unable to grip cage lid, 3 = unable to grip and has forelimb paralysis, and 4 = moribund (25).

Complement-dependent hemolytic assay

Blood was taken from the heart on the day of sacrifice with a syringe coated with heparin and kept at room temperature for 5 min before centrifugation at 8000 rpm for 5 min. Sera were assayed for complement-dependent hemolytic activity. Complement-dependent hemolytic assay was performed using Ab-sensitized sheep erythrocytes (Sigma-Aldrich) according to the manufacturer's instruction, with slight modification. Briefly, after several washes with gelatin veronal buffer (GVB^{2+}), the sheep erythrocytes (100 μl, 5×10^8/ml) were incubated at 37°C for 30 min with 50 μl of serially diluted sera in the presence of 50 μl of GVB^{2+} buffer, centrifuged at 2000 rpm for 10 min at 0°C, and OD_{541} of the supernatant fluid was read at 541 nm in a spectrophotometer. CH50 U, the volume of serum which gives 50% lysis, were calculated according to the manufacturer's instruction. The hemolytic activity correlates positively with serum dilution at which 50% lysis occurred, which is reciprocal of CH50 U.

Immunohistochemistry

The diaphragm was dissected and frozen in liquid N_2-cooled 2-methybutane and stored at −80°C until use. Ten-micrometer cryosections were acetone fixed for 5 min. After washing three times with PBS, the sections were blocked with 10% normal goat (or donkey, depending on the secondary Ab used) serum for at least 1 h. The sections were double labeled with Texas Red-labeled α-bungarotoxin (2 μg/ml; Molecular Probes) to identify endplates and goat anti-rat C3 (1/500; Cappel) or rabbit anti-rat C9 (1/500; a gift from Paul Morgan) (26). C3 or C9 was visualized with FITC-conjugated donkey anti-goat secondary Ab (1/500; Molecular Probes) or FITC-conjugated goat anti-rabbit secondary Ab (1/500; Molecular Probes). Sections were examined with a Nikon Diaphot fluorescence microscope (Nikon) and analyzed using ImagePro software (Media Cybernetics).

Ultrastructure of neuromuscular junctions

Electron microscopy was performed as described by Schneiter et al. (27), with exceptions as noted. After overnight fixation in 2% glutaraldehyde (with 0.1 M sodium cacodylate), the diaphragm was isolated at the synaptic region by cutting a 4-mm strip along the course of the phrenic nerve. The strip was washed and postfixed in 1% osmium tetroxide for 30 min. Muscle was transferred to 1% uranyl acetate for 1 h at room temperature, then dehydrated with ethanol and embedded in LX112 resin (Polysciences). Silver sections were cut and stained with 2% uranyl acetate followed by Reynolds lead citrate. Sections were examined with a JEM-100 CX11 electron microscope (JEOL) at 80 kV. Photographs were recorded using Kodak EM film (Eastman Kodak).

Methodological and statistical analysis

Ultrastructural, immunohistologic, and serologic studies were performed by laboratory personnel without prior knowledge of treatment or severity of weakness. Data were analyzed and tested for statistical significance ($p < 0.05$) using ANOVA and paired t tests. For comparison of C9 deposition, the pairwise nonparametric Kolmogorov-Smirnov two-sample test was performed.

Results

Anti-C5 mAb treatment prevents EAMG

In preliminary studies, we established a dose of anti-AChR mAb McAb-3 such that within 24 h of its administration, untreated rats developed hunched posture and dragged their legs while walking (grade 3 weakness) and within 48 h developed grade 4 weakness necessitating euthanasia (Fig. 1). With this dosage of anti-AChR mAb McAB-3, the rats exhibited chromodacryorrhea or "red tears," i.e., porphyrin deposits that are an indicator of stress (28). Having documented the effects of this dose in untreated rats, we next treated five rats with the same dose of anti-AChR mAb McAb-3 and anti-C5 mAb (0.03 mg/g rat). In marked contrast to

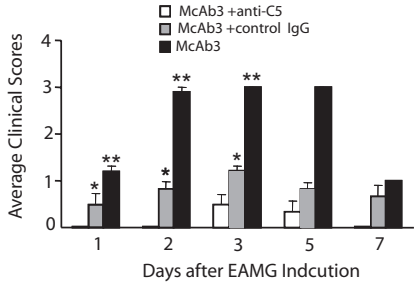

FIGURE 3. Clinical scores of EAMG rats treated with anti-C5 mAb or control IgG. Rats received anti-C5 mAb or control IgG 4 h before EAMG induction. Rats receiving McAb-3 alone ($n = 5$) developed EAMG on day 1, which became severe at day 2. In comparison, rats pretreated with anti-C5 mAb ($n = 6$) had clinical scores of 0 (**, $p < 0.0001$) at both days 1 and 2, whereas rats pretreated with control IgG Ab ($n = 6$) developed weakness of intermediate severity, which is significantly higher than anti-C5 mAb-treated rats ($p < 0.05$). On day 3, two of the six rats pretreated with anti-C5 mAb developed mild EAMG and recovered by day 5. Results are shown as means ± SE.

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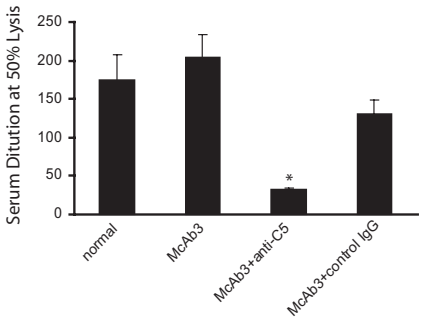

FIGURE 4. Anti-C5 mAb reduces serum hemolytic activity. The serum dilution at 50% hemolysis is shown and is calculated as the reciprocal of the CH50 U. The serum hemolytic activities of EAMG rats treated with anti-C5 mAb were significantly lower than those from normal rats, rats treated with control IgG, and EAMG rats without treatment (*, $p < 0.02$). There were no statistical differences among normal rats, rats treated with control IgG, and EAMG rats without treatment.

the untreated rats exposed to McAb-3, none of the five anti-C5 mAb-pretreated rats showed either muscle weakness or chromodacryorrhea during the 48 h of observation. One rat that was monitored for 7 days remained normal, with a clinical score of 0.

Anti-C5 mAb treatment inhibits EAMG progression after disease induction

In a subsequent set of experiments, we evaluated whether administration of the anti-C5 mAb to rats with existing disease would ameliorate weakness. For this, we induced EAMG with mAb McAb-3 on day 0 and administered the anti-C5 mAb 24 h later. At the time of anti-C5 mAb treatment, the two groups had equal strength scores (Fig. 2). In marked contrast, at 48 h the anti-C5 mAb-treated rats demonstrated striking improvement compared with controls. By 72 h while the control rats developed more severe EAMG (strength scores between 3 and 4) requiring euthanasia, the anti-C5 mAb-treated rats further improved and were normal by day 7.

Anti-C5 mAb effects are specific to complement inhibition

In a third set of experiments, we used a mAb which is directed against rat C5 but does not inhibit C5 hemolytic activity (24). We pretreated groups of rats with 1) this non-neutralizing anti-C5 mAb, 2) the above neutralizing anti-C5 mAb, or 3) no mAb, and 4 h later administered (the same does of) McAb-3. At 24 h after McAb-3 administration, the non-neutralizing anti-C5 mAb-treated rats developed weakness with clinical scores significantly greater than the neutralizing anti-C5 mAb-treated rats, all of which appeared normal (Fig. 3). At 48 and 72 h, the difference widened. With the exception of one animal, sham-treated rats required euthanasia on day 3. Although only one neutralizing anti-C5 mAb-treated rat showed minimal weakness which resolved by day 7, three control IgG non-neutralizing anti-C5 mAb-treated rats continued to show weakness on day 7.

The neutralizing anti-C5 mAb depleted serum C5 activity

For each of the above experiments, we performed hemolytic assays with Ab-sensitized sheep erythrocytes ($E^{sh}A$) to verify the extent to which anti-C5 mAb depleted C5. These assays showed that the

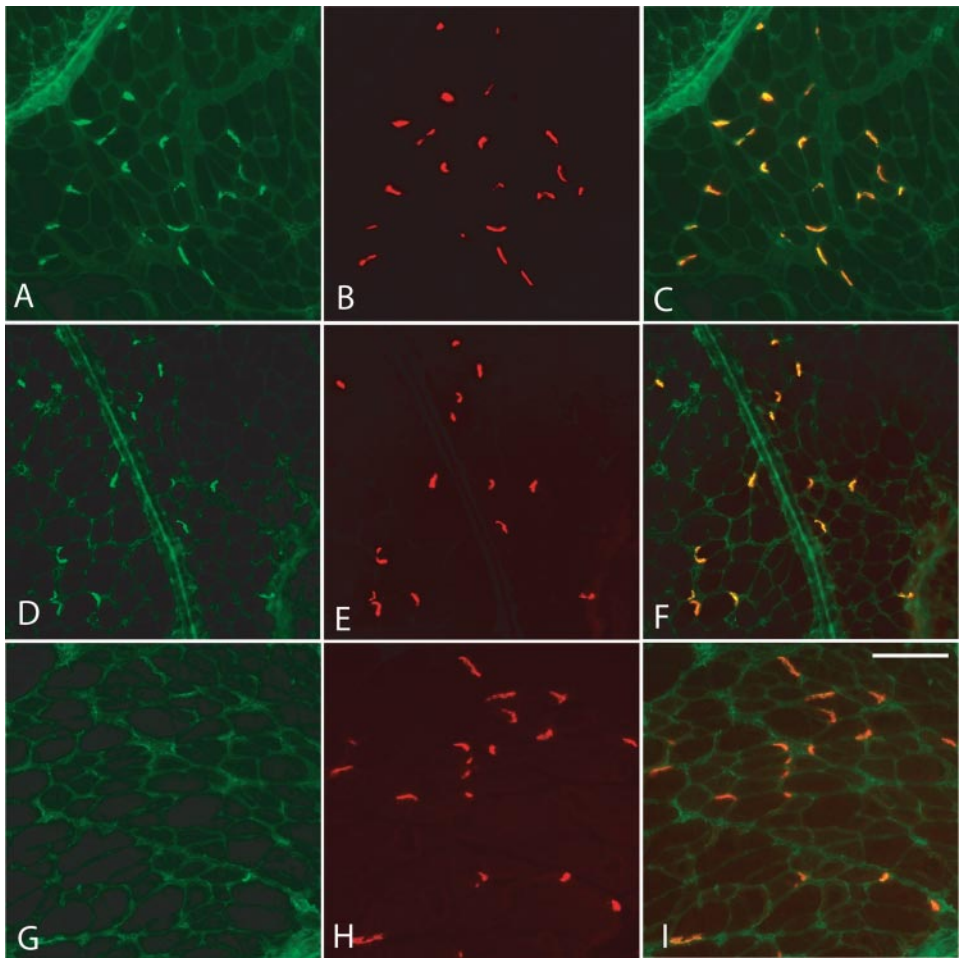

FIGURE 5. Anti-C5 mAb treatment attenuates C9 deposition at the neuromuscular junction. The muscle sections of EAMG rats with different treatments were doubly labeled with rabbit anti-C9 (green, *A*, *D*, and *G*) and Texas Red-labeled α-bungarotoxin to identify endplates (red, *B*, *E*, and *H*). *C*, *F*, and *I*, The merged composite of red and green images to show the localization of C9 deposition at the neuromuscular junction. *A–C,* From muscles of EAMG rats without treatment; *D–F,* from control IgG-treated rats; and *G–I,* from anti-C5 mAb-treated rats. The muscles all came from rats 48 h after EAMG induction. C9 immunoreactivity was concentrated at curved edges of muscle fibers in the typical shape of a neuromuscular junction. C9 immunoreactivity is much weaker from anti-C5 mAb-treated rats compared with untreated and control IgG-treated rats. Scale bar, 100 μm for all images.

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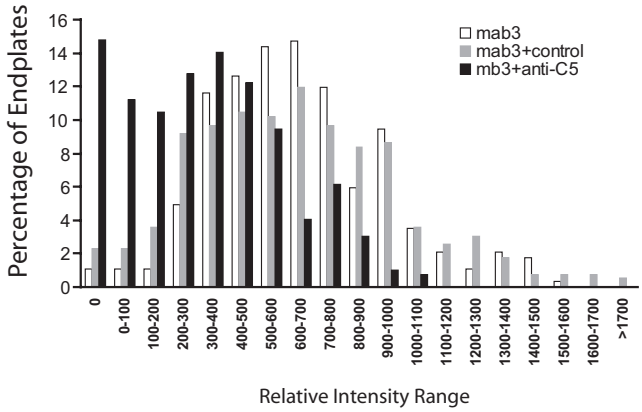

FIGURE 6. Anti-C5 mAb treatment reduced the intensity of C9 immunofluorescence at the neuromuscular junction. This graph shows the percentage of neuromuscular junctions within certain fluorescence intensity range from EAMG rats, EAMG rats treated with anti-C5 mAb, and EAMG rats treated with control IgG. The intensities of junctions from anti-C5 mAb-treated rats were shifted to the lower intensity ranges and is significantly weaker than the other two groups ($p < 0.0001$ for the nonparametric Kolmogorov-Smirnov two-sample test). There is no significant difference in the distribution of fluorescence intensity between the junctions of untreated and control IgG-treated rats.

single injection of neutralizing anti-C5 mAb totally abolished serum C5 activity 48 and 72 h after administration (Fig. 4). By day 7 however, C5 activity had returned to normal (data not shown). In rats receiving McAb-3 alone, no alteration of C5 activity occurred. Similarly, the non-neutralizing anti-C5 mAb did not inhibit C5 activity. Only those rats treated with the neutralizing anti-C5 showed complete elimination of C5 activity.

The neutralizing anti-C5 mAb reduces C9 but not C3b deposition

Immunofluorescence staining of neuromuscular junctions of EAMG muscles showed that treatment with the neutralizing anti-C5 mAb reduced C9 deposition (Fig. 5), whereas treatment with the non-neutralizing control IgG mAb did not. Pixel quantitation of the C9 deposition (Fig. 6) showed that muscles from the untreated and non-neutralizing anti-C5 mAb-treated rats contained a higher percentage of neuromuscular junctions with high-density C9 deposits than muscles from IgG-neutralizing anti-C5 mAb-treated rats. C3 immunoreactivity at the neuromuscular junction was similar in intensity in all groups, consistent with inhibition of the complement cascade at a step subsequent to activation of C3.

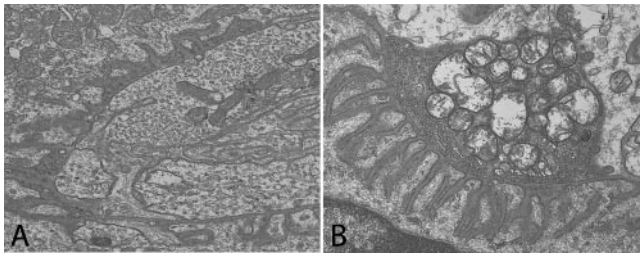

FIGURE 7. Anti-C5 mAb treatment preserves the integrity of the neuromuscular junctions. Electrical microscopy was performed to study the structure of neuromuscular junctions of diaphragms of EAMG rats. The neuromuscular junctions from untreated EAMG rats (*A*) were abnormal. They either had shortened or no junctional folds, widened junctional space, widened synaptic cleft, or electron-dense spots that contained AChR-rich membrane. The neuromuscular junctions from anti-C5 mAb-treated rats (*B*), in contrast, had relatively normal junctional folds and space and normal synaptic cleft with some membrane blebbing.

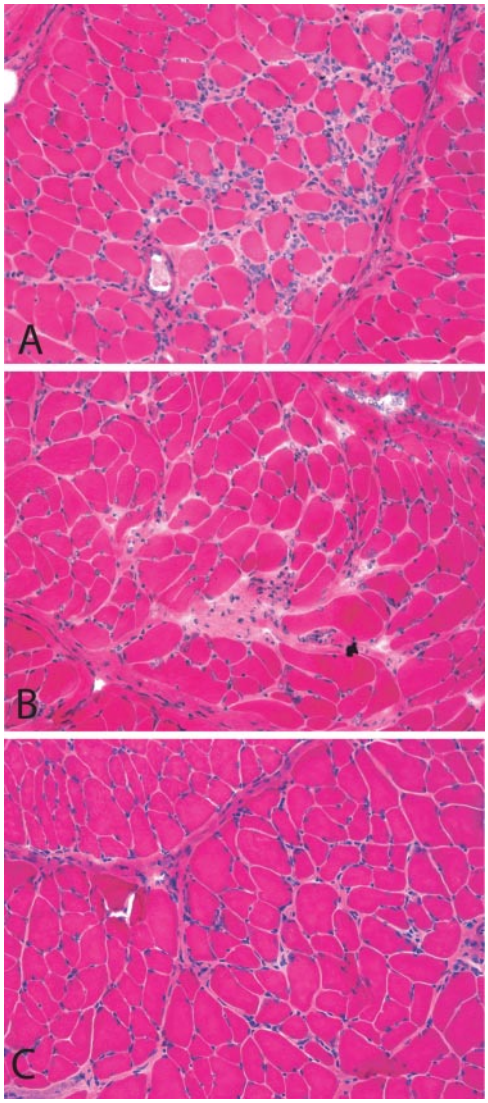

FIGURE 8. Anti-C5 mAb treatment reduced muscle inflammation. Panels are H&E-stained muscles of untreated (*A*), control Ab-treated (*B*), and anti-C5 mAb-treated rats (*C*). The muscle sections are from day 2 EAMG rats. Inflammation in EAMG rats varies from area to area even within the same muscle type. Anti-C5 mAb treatment moderated inflammation compared with untreated and control Ab-treated rats.

Anti-C5 mAb prevents disruption of neuromuscular junctional architecture

Ultrastructural analysis of neuromuscular junctions from diaphragms of rats that did not receive the neutralizing anti-C5 mAb showed simplification of junctional folds, widened synaptic clefts, with electron-dense material consistent with sloughed synaptic membrane (Fig. 7). In contrast to these typical findings of severe EAMG (4), neuromuscular junctions from diaphragms of rats treated with the neutralizing anti-C5 mAb showed only minimal changes (Fig. 7).

Anti-C5 mAb treatment reduces muscle inflammation

Inflammatory infiltrates composed of monocytes and macrophages are typical features of passively induced EAMG (4, 12). Fig. 8 shows representative sections of diaphragm muscles of EAMG

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rats. Diaphragm sections from rats treated with the neutralizing anti-C5 mAb showed significantly lower numbers of inflammatory cells compared with sections from untreated and IgG control non-neutralizing anti-C5 mAb-treated rats.

Discussion

In EAMG, injury to neuromuscular junctions is caused primarily by AChR autoantibody initiation of autologous complement activation on motor endplates. In this study, we found that functional blockade of C5 with the neutralizing anti-C5 mAb completely prevented weakness in EAMG and that depletion 24 h after disease initiation abrogated disease progression. Administration of an isotype control Ab directed at C5 that does not inhibit C5 function (control IgG) was not effective at preventing weakness, verifying that circumvented neuromuscular junction damage was not due to simple binding of the mAb to C5 or to the inhibitory effects of Ig on complement activation. Serum hemolytic assays verified that C5b-9- dependent hemolytic activity was markedly reduced by the neutralizing anti-C5 mAb. Consistent with the preservation of strength in anti-C5 mAb-treated rats, neuromuscular junction architecture was maintained. C9 deposition at neuromuscular junctions was significantly reduced. In accordance with anti-C5 mAb acting downstream of C3, deposited C3b in neuromuscular junctions of neutralizing anti-C5 mAb and IgG control non-neutralizing anti-C5 mAb-treated junctions was comparable.

The characteristic disease course of passively induced EAMG in rats is that weakness peaks 48–72 h after induction and then gradually resolves (29, 30). In our experimental protocol, nearly all untreated EAMG rats required euthanasia 48–72 h after the AChR Ab administration, but in all cases the neutralizing anti-C5 mAb saved them. The non-neutralizing anti-C5 mAb moderated disease severity but not to the level observed for the anti-C5 mAb.

In passively acquired EAMG, an extensive inflammatory infiltrate forms which is due to the chemotactic effects of C5a and elaborated cytokines. e.g., IL-8 once complement activation has occurred (4, 12). In the current study, the neutralizing anti-C5 mAb reduced the inflammatory infiltrate. In published studies of anti-C5 mAb therapy in myocardial infarction and reperfusion injury (24), polymorphonuclear leukocyte infiltration was reduced, verifying that C5a plays a major role in recruiting these cells to injured muscle. By contrast, rats deficient in C6 or treated with anti-C6 Ab do not have reduced inflammatory infiltration, presumably due to the activity of C5a (12). The current study identified that the non-neutralizing control mAb moderated the severity of EAMG weakness, in contrast to a previous report using this same mAb, which had failed to identify a functional effect (24). The anti-C5 mAb that blocks cleavage and C5 functions both to prevent terminal pathway progression to form the MAC and prevent the generation of C5a, whereas the anti-C5 control mAb, by virtue of binding to C5, may be sufficient to decrease disease activity, but not to significantly block hemolytic activity or C9 deposition. However, we are not certain as to the precise mechanism by which the control Ab produced disease moderation. It could have been due to the fact that 1) the total hemolytic assay does not directly measure C5 and that C5 was depleted to an extent to produce a therapeutic effect; 2) that the ameliorating effect is Fc mediated, a phenomenon that is well established in the literature (31); or 3) that some other mechanism may be involved such as reduced internalization of the AChR due to binding to C5 deposited on it.

Extensive studies of EAMG and, to some extent human MG, suggest that complement plays a primary role in the disease. Ultrastructural studies in the human disease have identified dense MAC deposits and MAC-positive debris at the neuromuscular junction (4, 8, 9). Although passive EAMG does not recapitulate the breakdown of self-tolerance in human MG, it accurately reproduces a common pathway of autoantibody-initiated, complement-mediated destruction of the neuromuscular junction. There are some patients that demonstrate an extremely rapid response to plasma exchange treatment, which would suggest that removal of Ab that blocks AChR ion channel function is a non-complement-mediated mechanism of disease in humans (1).

The postsynaptic portion of the neuromuscular junction in human MG and EAMG induced either by AChR immunization or by administration of AChR Abs is similar. Our finding that C5 blockade protects from development of severe EAMG provides strong support that complement inhibition is a viable treatment approach for human MG. Our findings thus argue that inhibition of complement with a functionally blocking anti-C5 mAb can be an effective therapy, especially in situations of severe weakness, such as myasthenic crisis.

Disclosure

Russell P. Rother is employed by Alexion Pharmaceuticals.

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