Article

The Effect of CD3-Specific Monoclonal Antibody on Treating Experimental Autoimmune Myasthenia Gravis

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CD3-specific monoclonal antibody was the first one used for clinical practice in field of transplantation. Recently, renewed interests have elicited in its capacity to prevent autoimmune diabetes by inducing immune tolerance. In this study, we tested whether this antibody can also be used to treat another kind of autoimmune disease myasthenia gravis (MG) and explored the possible mechanisms. MG is caused by an autoimmune damage mediated by antibody- and complement-mediated destruction of AChR at the neuromuscular junction. We found that administration of CD3-specific antibody (Fab)$_2$ to an animal model with experimental autoimmune myasthenia gravis (EAMG) (B6 mice received 3 times of AChR/CFA immunization) could not significantly improve the clinical signs and clinical score. When the possible mechanisms were tested, we found that CD3 antibody treatment slightly down-regulated the T-cell response to AChR, modestly up-regulation the muscle strength. And no significant difference in the titers of IgG2b was found between CD3 antibody treated and control groups. These data indicated that CD3-specific antibody was not suitable for treating MG, an antibody- and complement-mediated autoimmune disease, after this disease has been established. The role of CD3-specific antibody in treating this kind of disease remains to be determined. ***Cellular & Molecular Immunology*****. 2005;2(6):461-465.**

Key Words: CD3-specific monoclonal antibody, immune tolerance, EAMG

Introduction

Myasthenia gravis (MG) and experimental autoimmune myasthenia gravis (EAMG) are antibody-mediated, T-cell-dependent autoimmune diseases in which the nicotinic acetylcholine receptor (AChR) at the neuromuscular junction is the major auto-antigen. Antibody- and complement-mediated destruction are the primary mechanisms involved in the destruction of AChR (1). EAMG is similar to the human disease in its clinical and immunopathologic manifestations and presents a widely used model for the investigation of new therapeutic strategies (2). In clinical trails, the routine therapies of MG include prednisone and thymectomy as well as inhibition of acetylcholinesterase. Although these strategies permitted substantial control of MG and have greatly reduced its mortality (3), the significant side effects are associated with prolonged nonspecific immune suppression. Thus, how to induce antigen-specific tolerance became the focus for many immunologists.

CD3-specific monoclonal antibody as a potent immuno-suppressant has been widely used in clinic for a few years. Good results have also been achieved in transplant and IDDM (4). CD3-specific antibodies are antigen-non-specific, nevertheless promote a long-lasting antigen-specific effect. Therefore it provides a new way for the treatment of autoimmune disease. In this study, we treated EAMG by CD3-specific monoclonal antibody, and explored the possible mechanisms, hoping to find new approaches to treat this kind of autoimmune disease.

Materials and Methods

Animals and antigen preparation

Female B6 mice, 8-10 weeks of age, were obtained from Charles River Co. All animals were bred in our animal facilities under specific pathogen-free conditions. All the experiments performed for this study were according to the institutional guideline for animal care. AChR was derived from the electric organ of Torpedo as described (5).

Monoclonal antibodies

Goat anti-mouse IgG2b(H) mAb, HRP-rabbit anti-goat IgG (H + L) mAb, were purchased from Sigma Company. CD3-specific monoclonal antibody (Fab)$_2$ was provided by Beijing Tian Guang Shi Company. It is rodent rat-antimouse antibody

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and with no mitogenic potential.

Induction and clinical evaluation of EAMG

For *in vivo* studies, all mice were anesthetized and immunized with 0.8 mg AChR emulsified in CFA (Sigma) *s.c.* at four sites, two hind footpads and shoulders on day 0. Mice were boosted on day 28 and day 56, and were monitored on alternate days and the clinical severity was graded between 0 and 4, where grade 0 stands for healthy normal mice with no symptoms of EAMG; grade 1, mildly decreased activity, weak grip, with fatigability; grade 2, weakness, hunched posture at rest, decreased body weight and tremor; grade 3, severe generalized weakness, significant decrease in body weight, moribund; grade 4, dead (6). Clinical EAMG was also confirmed by *i.p.* administration of 50 μl neostigmine bromide (15 μg/ml) together with atropine sulfate (6 μg/ml) in PBS, and observing improvement in muscle strength (7). Clinical grades were recorded weekly in a double blind evaluation method for 10-12 weeks following the immunization with Torpedo AChR.

ELISA for determining IgG2b of anti-AchR antibody

Sera from day 56 and day 70 were tested to determine a potential difference between the two groups regarding the IgG2b isotype of anti-AchR antibody according to previously described method (8). Briefly, microtiter plates were coated with 100 μl per well of AChR (2 μg/ml) at 4°C overnight and uncoated sites were blocked with 10% fetal calf serum. Dilute serum with a pre-determined amount of anti-AChR antibodies was added and incubated for 2 h at room temperature. Then, plates were incubated for 2 h with goat anti-mouse IgG2b, followed by HRP-conjugated rabbit anti-goat Ab. Results were expressed as antibody titer.

Lymphocyte proliferation response

Splenocytes from treated and untreated groups were collected on day 70, and single-cell suspensions (100 μl) were applied in triplicate into round-bottomed 96-well microtiter plates at a cell density of 5×10^5/ml. Ten-μl aliquots of either AChR or OVA (25 μg/ml) were added into wells. After 56-hour incubation, the cells were pulsed for 16 h with 10-μl aliquots containing 1 μCi of (^{3}H) methylthymidine. Cells were harvested onto glass fiber filters and thymidine incorporation was measured. The results were expressed as stimulation index (SI), which was calculated by dividing the cpm from culture in the presence of antigen or mitogen by the cpm from culture without antigen.

Measurement of electromyography

Mice were anesthetized by intraperitoneal administration of ketamine (200 mg/g body wt). After the anesthetization, mice were restrained on a cork dissecting board, the hip was shaved and a topical disinfectant was applied, a 1.5-cm midline incision was made, the gastrocnemius and the sciatic nerve was identified. Paired stimulating electrodes separated by 2-3 mm were inserted intramuscularly near the sciatic notcl. Recorded electrodes were inserted in the medial compartment of the gastrocnemius, and electromyographic responses were recorded by consecutively incentiving the sciatic nerve at intensity of 5HE, 1.5mA. The results were reflected by rate of attenuation of the gastrocnemius combined action potential.

Statistical analysis

To determine the significance of the observed results, two statistical tests were used. Incidence of EAMG was compared using the Fisher's exact test. Antibody titers were compared using Student's *t*-test. Values of $p < 0.05$ were accepted as indicating significance. The antibody titer was demonstrated by scatter diagram.

Results

Effects of CD3-specific antibody treatment on preventing EAMG

To test the possibility of CD3-specific antibody in treating AChR-induced EAMG in susceptible C57BL/6 mice, we immunized C57BL/6 (n = 32) mice with AChR/CFA on day 0 and boosted on days 28 and 56. After the second boost, the mice were divided into two groups (n = 16) at random, and were injected either with CD3-specific monoclonal antibody (5 μg/mouse) or with PBS control for five days. The kinetics on the incidence of clinical EAMG is illustrated in Table 1. The difference in the incidence of disease between antibody-treated group and PBS control was not significant from day 56 to termination of mice. Seven of sixteen antibody-treated mice were diagnosed with disease, and six of sixteen PBS-treated mice. Mice with grade 1 or more were injected with neostigmine in combination with atropine sulfate. All mice with muscle weakness showed temporary improvement soon after administration of neostigmine in combination with atropine sulfate.

Determining IgG2b of anti-AchR antibody

To study the effect of CD3-specific antibody administration, we analyzed the IgG2b in the sera, which was an important Ab isotype in inducing autoimmune damage in MG. Blood samples were collected at the second boost (d = 56) and day 70 following disease induction. The analyses of IgG2b

Table 1. Effect of CD3-specific antibody on treating ongoing EAMG

Group	Severity of muscle weakness Grade					Mice with weakness (%)
	0	1	2	3	4	
Antibody-treated	9	5	1	-	1	7/16 (43.75)
PBS	10	5	1	-	-	6/16 (37.5)

Percentage of mice with clinical signs of EAMG and clinical score. Torpedo AChR was injected to induce EAMG, and mice were treated five days by administration of CD3-specific antibody or PBS. Incidence of EAMG was compared using Fisher's exact test. $p > 0.05$ at termination.

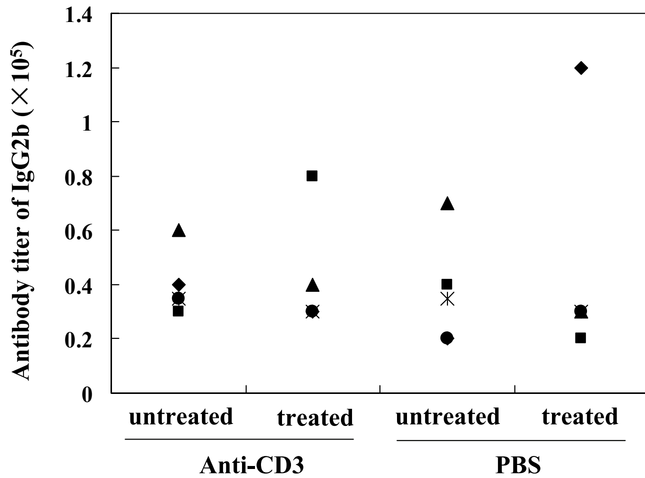

Figure 1. The titers of IgG2b antibody before and after treatment. Serum was collected from antibody-treated group and PBS control by tail bleeding, 56 days and 70 days after immunization. Anti-AChR IgG2b was determined using ELISA on Torpedo-AChR coated plates as described in "**Materials and Methods**". Untreated means before the antibody or PBS treatment, Treated means after the antibody or PBS injection (n = 5).

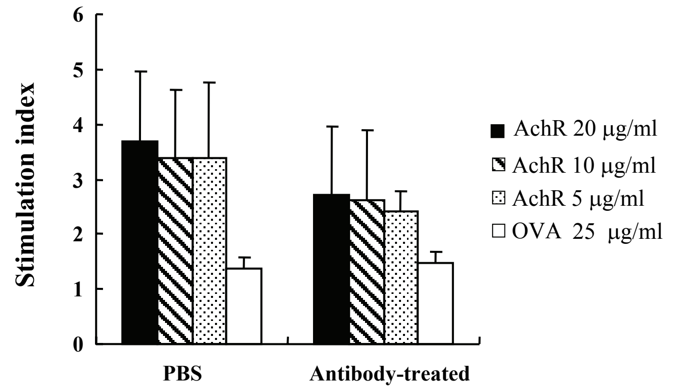

Figure 2. Proliferation response of splenocytes derived from antibody-treated and PBS control group. Splenocytes from two groups were processed into a single-cell suspension and challenged *in vitro* with different doses of AchR for 3 days. Proliferative responses were studied on 70 days after immunization, when EAMG was evident.

elicited after treatment with antibody or PBS control revealed that IgG2b, regulated by Th1 cells, was not significantly down-regulated in mice treated with the CD3-specific antibody (Figure 1). After administration with antibody or PBS control, all groups of mice showed elevated serum levels of IgG2b detected by ELISA, the antibody titer of antibody-treated group was increased from 1:40,000 to 1:42,000, and that of the PBS group was increased from 1:37,000 to 1: 46,000. No significant difference between the two groups was found ($p > 0.05$).

Lymphocyte proliferation response

Previous study showed that CD3-specific antibody treatment can induce immune tolerance in renal-allograft recipients. Here we tested this phenomenon using *in vitro* T cells proliferation assay. To study the effect of CD3-specific antibody administration on cellular AChR-specific responses, we measured the *in vitro* response to AChR of splenocytes from treated or control mice. Splenocytes were aseptically removed and cultured in the presence of different doses of Torpedo AChR. Compared with PBS-injected control, mice receiving antibody at dose of 5 μg/day had lower proliferative responses to AChR on examination of splenocytes 70 days after immunization with AChR + CFA. But no significant difference ($p > 0.05$) was detected between the two groups. In control experiment, splenocytes were cultured in the presence of the control antigen OVA. Control experiment is the cultures of splenocytes in the presence of the control antigen OVA. Figure 2 summarizes the average of the stimulated index (SI) in three independent proliferation assays.

Measurement of electromyography

Previous data have shown that myasthenic mice exhibit electromyography similar to those seen in human myasthenics, the compound muscle action potential of the myasthenic mouse decrement upon stimulation. So we tested the possibility that treatment of myasthenic mice with CD3-specific antibody could result in improvement of decrement. When comparing the antibody-treated group and PBS control with normal mice, electromyogram showed reduced amplitude of gastrocnemius combined action potential, but a little difference between the antibody treated group and PBS control still could be seen. The rates of attenuation in the antibody-treated mice are higher than those

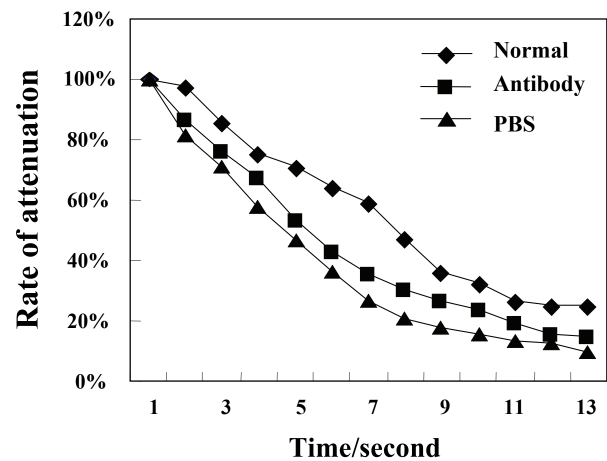

Figure 3. Amplitude of gastrocnemius combined action potential after 5HE, 1.5mA stimulation. After anesthetization, mice were restrained on a board, and the rate of attenuation was recorded by persistent incentive the sciatic nerve. We observed the response 70 days after the first immunization.

in PBS control.

Discussion

Myasthenia Gravis is a common disease which was associated with immune dysfunction in neuro-immune system. The hallmark of an autoimmune neuromuscular disorder in MG is the loss of functional AChR at the postsynaptic membrane, mediated by anti-AChR antibodies and complement. The autoimmune destruction of AChR results in a defective neuromuscular transmission causing muscle weakness and fatigue characteristic of MG (9). With the deeper understanding of the pathogenesis, the novel progress has been made. In clinical trails, the methods including steroid and thymectomy have achieved some effects, but it can't reverse the pathological course. How to reconstruct the normal immune system becomes the focus of the therapy. Because it has been argued that MG is the best characteristic autoimmune disease, successfully specific immuno- suppression of MG might be valuable not only for its benefit to MG patients, but also as a model for what might be applied to other autoimmune disease with well characterized antigens.

Ideal treatment of autoimmune disease should inhibit the immune response against target antigens specifically and permanently without interfering with the general function of the immune system (10). When compared with the conventional immunosuppressant, CD3-specific monoclonal antibody has some advantages, with few side-effects and long-term maintenance, so it was widely used in clinic. At the same time, with the capacity of inducing antigen-specific tolerance, the antibody turns into a focus in the field, and the property has been widely identified in clinical treatment of IDDM (11). The effects comprise two consecutive phages. The first phage is to clear the activated pathogenic T cells, and also associated with a transient Th2 polarization. The hallmark of the second phage, long-time phage, is the coexistence of pathogenic T cells and TGF-β-depend regulatory T cells (12). As indicated in elsewhere (13), by whatever mechanisms the autoimmune response might be initiated in the various forms of MG, all autoimmune forms shared the final common pathway of a T-cell-dependent antibody-mediated autoimmune response to muscle AChR. The CD3-specific monoclonal antibody is mainly targeted the activated pathogenic T cells, but during the course of MG, the antibody- and complement-mediated destruction are the primary mechanisms involved in the destruction of AChR, so the possibility of the therapy is the first important question that should be concerned.

We repeated the animal model of MG by AChR which was extracted from Torpedo electroplax tissue. During the process we administrated CD3-specific monoclonal antibody; subsequently, we evaluated the result by observing the titer of IgG2b antibody, which was highly associated with deteriorism of the autoimmune disease (13). It also reflected the property of antibody administration in down-regulation of Th1 to Th2 regulation, because involvement of Th1 cytokines in the pathogenesis of autoimmune disease is widely accepted, but we couldn't see significant difference before and after treatment. We proposed that the IgG2b antibody is emerged before the administration, and the antibody-mediated destructive effect has already existed, thus the treatment of CD3-specific monoclonal antibody couldn't reverse the pathological progress. In the same time, we studied the proliferation of the activated pathogenic T cells *in vitro*, which reflect the capacity of CD3-specific monoclonal antibody inducing antigen-specific tolerance, but the antibody didn't induce tolerance of activated pathogenic T cells to self-antigen AChR. It is possible that, CD3-specific antibody injection targeted the newly activated pathogenic T cells, as proved in treating IDDM, that administration of CD3-specific antibody in the course of MG couldn't tolerate the pathogenic T cells. It was also possible that, after the large scale destruction of muscular cytosolic proteins, the CD3-specific antibody has no ability to induce tolerance to so many series of antigens, such as actin, myosin, α actinin, titin, rapsyn, et al. Therefore the T cell proliferation to self-antigen is still maintained. We also observed the gastrocnemius combined action potential, which is an objective, quantitative measurement of muscle weakness. Because no significantly decremental response to repetitive stimulation was found in any control animal which had not developed clinical signs of EAMG, and decremental electromyography patterns were demonstrated at the peak of clinical weakness. On the contrary, the decrement lessened with clinical signs of improvement. Nevertheless, the outcome is not as satisfied as we expected, the decremental response to repetitive nerve stimulation isn't changed, which implicated that CD3-specific antibody couldn't reverse the pathological progress and improve the muscle strength.

More than 20 years have passed since CD3-specific monoclonal antibodies were first administrated in clinic. In treating IDDM, CD3-specific antibodies can induce stable disease remission by restoring long-time tolerance to pancreatic β-cells. But in our experiment, the administration of CD3-specific antibodies ultimately couldn't acquire the expected result, in spite of some trends of tolerance, the response to auto-antigen AChR is still in existence, and the muscle strength is not improved. But other factors should be considered, the dose of antibody and the intervention timepoint are all might be associated with the outcome. Recently, systemic or oral tolerance to AChR or its dominant peptides has achieved great progress, and down- regulation of pathogenic cytokines becomes effective therapy of MG and its animal model EAMG. Thus applying CD3-specific antibody to current MG therapy still needs further investigation.

References

1. Feferman T, Im SH, Fuchs S, et al. Breakage of tolerance to hidden cytoplasmic epitopes of the acetylcholine receptor in experimental autoimmune myasthenia gravis. J Neuroimmunol. 2003;140:153-158.

2. Baggi F, Annoni A, Ubiali F, et al. Break of tolerance to a self-peptide of acetylcholine receptor α-subunit induces experimental myasthenia gravis in rats. J Immunol. 2004;172: 2697-2703.
3. Christadoss P, Poussin M, Deng C. Short analytical review: animal model of muasthenia gravis. J Clin Immunol. 2000;94: 75-87.
4. Ortho Multicenter Transplant Study Group. A randomized clinical trail of OKT3 monoclonal antibody for acute rejection of cadaveric renal transplants. N Engl J Med. 1985;313:337-342.
5. Aharonov A, Tarrab HR, Silman R, et al. Immunochemical studies on acetylcholine receptor fraction from Torpedo califoration. Immunochemistry. 1977;14:129-139.
6. Maiti PK, Feferman T, Im SH, et al. Immunosuppression of rat myasthenia gravis by oral administration of a syngeneic acetylcholine receptor fragment. J Neuroimmunol. 2004;152: 112-120.
7. Wu B, Deng CS, Goluszko E, et al. Tolerance to a dominant T cell epitope in the acetylcholine receptor molecule induces epitope spread and suppresses mnrine myasthenia gravis. J Immunol. 1997;159:3016-3023.
8. Zhang GX, Xiao BG, Yu LY, et al. Interleukin 10 aggravates experimental autoimmune myasthenia gravis through inducing Th2 and B cell response to AChR. J Neuroimmunol. 2001;113: 10-18.
9. Dedhia V, Goluszko E, Wu B, et al. The effect of B cell deficiency on the immune response to acetylcholine receptor and the development of experimental autoimmune myasthenia gravis. Clin Immunol Immunopathol. 1998;87:266-275.
10. Baggi F, Andreetta F, Caspani E, et al. Oral administration of an immunodominant T-cell epitope downregulates Th1/Th2 cytokines and prevents experimental myasthenia gravis. J Clin Invest. 1999;104:1287-1295.
11. Chatenoud L. CD3-specific antibody-induced active tolerance: from bench to beside. Nat Rev Immunol. 2003;3:123-132.
12. Chatenound L, Thervet E, Primo T, et al. Anti-CD3 antibody induces long-term remission of overt autoimmunity in nonobese diabetic mice. Proc Natl Acad Sci U S A. 1994;91:123-127.
13. Li Z, Forester N, Vincent A, et al. Modulation of acetylcholine receptor function in TE671 cells by non-AChR ligands: possible relevance to seronegative myasthenia gravis. J Immunol. 1996; 64:179-183.