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Experimental autoimmune myasthenia gravis induction in B cell-deficient mice

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Abstract

Experimental autoimmune myasthenia gravis (EAMG) is an animal model for human myasthenia gravis (MG). Autoantibody-induced functional loss of nicotinic acetylcholine receptor (AChR) at the postsynaptic membrane is an important pathogenic feature of both MG and EAMG. To evaluate the extent at which the humoral immune response against AChR operates in the pathogenesis of EAMG, we immunized B cell knockout (μMT) and wild-type C57BL/6 mice with AChR and complete Freund's adjuvant. The ability of AChR-primed lymph node cells to proliferate and secrete IFN-γ in response to AChR and its dominant peptide α146–162 were intact in μMT mice as in wild-type mice. Similar amounts of mRNA for IFN-γ, IL-4 and IL-10 in AChR-reactive lymph node cells were detected in μMT and wild-type mice. However, μMT mice had no detectable anti-AChR antibodies and remained completely free from clinical EAMG. We conclude that B cells are critically required for the genesis of clinical EAMG, but not for AChR-specific T cell priming.

Introduction

Myasthenia gravis (MG) is a classic antoimmune disease affecting neuromuscular junctions of striated muscle. Immunization of different animal species with acetylcholine receptor (AChR) and complete Freund's adjuvant (CFA) results in an animal model of MG named experimental autoimmune myasthenia gravis (EAMG). The prevailing notion is that the impaired neuromuscular transmission in both MG and EAMG is caused by antibodies against AChR (1–3). Several lines of evidence suggest that cell-mediated immune effector responses may also participate in the pathogenesis of MG and EAMG. Firstly, there is no direct correlation between the anti-AChR antibody levels and the clinical severity of both human MG and EAMG (2–5). Between 10 and 15% of patients with MG have no detectable anti-AChR antibodies in their serum (3). Secondly, focal accumulations of mononuclear cells have been observed in striated muscle specimens from MG patients and EAMG rats (6–9). Thirdly, antibody- and complement-dependent cell-mediated cytotoxicity does occur in EAMG in rat (8,9).

The production of anti-AChR autoantibodies is dependent on T cell help (10–12). In the immunopathogensis of MG and EAMG, B cells are expected to serve as antigen-presenting cells (APC) for autoreactive T cell priming and as effector cells that secrete anti-AChR antibodies. To evaluate the extent at which abnormal B cell responses against AChR operate in the pathogenesis of EAMG, we compared the development of EAMG in susceptible C57BL/6 (B6, H-2^b haplotype) mice (4) and in B6 mice rendered deficient of B cells (μMT). We found that μMT mice were completely resistant to EAMG, as compared to wild-type control mice. B cells are thus essentially required for the genesis of EAMG in mice, while the immune effector role of other cell types, if there is any, seems not to be sufficient for the genesis of EAMG.

Methods

Mice

B6 μMT mice, originally obtained from B & K (Universal, Hull, UK), were generated by targeted disruption of the μ heavy chain transmembrane exon (13). Inbred B6 mice were obtained from Bommice (Ry, Denmark) and used as a wild-type control. Mice were bred and maintained under pathogen-free conditions in the animal facilities of the Huddinge Univer-

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sity Hospital. All mice used were females and 8–12 weeks of age. Animal experimental procedures were in compliance with institutional guidelines.

Antigens and synthetic AChR α chain peptide

AChR was purified from the electric organs of *Torpedo* Californica (Pacific Biomarine, Venice, CA) by affinity chromatography on an α-cobrotoxin–agarose resin (Sigma, St Louis, MO) (14). The isolated product was pure as judged by SDS–PAGE. The purified *Torpedo* AChR was used to induce EAMG and for *in vitro* culture. Mouse muscle AChR (M-AChR) was extracted from normal B6 mouse (14) for use as antigen to detect anti-mouse AChR antibodies. Myelin basic protein (MBP) for use as control antigen was purified from normal mouse brains (15). Synthetic dominant peptide (α146–162) AChR α chain (16): L-G-I-W-T-Y-D-G-T-K-V-S-I-S-P-E-S was used for *in vitro* culture.

Induction and clinical evaluation of EAMG

B6 and μMT mice were immunized s.c. along the shoulders and back with 20 μg AChR with CFA in a total volume of 100 μl, and boosted twice at monthly intervals with 20 μg of AChR in CFA s.c. at four sites on the shoulders and thighs. The mice were observed every other day in a blinded fashion for signs of muscle weakness characteristic of EAMG. The clinical symptoms were graded between 0 and 3 (4): 0, no definite muscle weakness; 1+, normal strength at rest but weak with chin on the floor and inability to raise the head after exercise consisting of 20 consecutive paw grips; 2+, as grade 1+ and weakness at rest; and 3+, moribund, dehydrated and paralyzed. Clinical EAMG was confirmed by injection of neostigmine bromide and atropine sulfate (4).

Lymphocyte proliferation responses

μMT and wild-type B6 mice, four to six in each group, were immunized with 20 μg *Torpedo* AChR in CFA s.c. in the hind footpads and thighs. Seven days later, mice were killed and mononuclear cell (MNC) suspensions from the popliteal and inguinal lymph nodes (PILN) were prepared (17). Triplicate aliquots (200 μl) of MNC suspensions containing 4×10^5 cells were applied into 96-well round-bottomed microtiter plates (Nunc, Copenhagen, Denmark). Then 10 μl aliquots of either AChR, α146–162 peptide, MBP or concanavalin A (Con A) were added into appropriate wells at a final concentration of 10 μg/ml (AChR, α146–162 peptide or MBP) or 5 μg/ml (Con A). After 4 days of incubation, the cells were pulsed for 18 h with 10 μl aliquots containing 1 mCi of [^{3}H]methylthymidine (sp. act. 42 Ci/mmol; Amersham, Arlington Heights, IL). Cells were harvested onto glass fiber filters and thymidine incorporation was measured.

Enumeration of AChR and α146–162 reactive IFN-γ secreting cells

A solid-phase enzyme-linked immunospot (ELISPOT) assay was adopted (17–19). Nitrocellulose-bottomed microtiter plates were coated with 100 μl IFN-γ capture antibody (DB1; Innogenetics, Genth, Belgium) at 15 μg/ml. Aliquots of 200 μl of cell suspension containing 4×10^5 MNC were added to individual wells in triplicate, followed by antigen (AChR or α146–162 or MBP) or the mitogen Con A (Sigma) in 10 μl aliquots to a final concentration of 10 μg/ml of AChR or MBP or peptide, or 5 μg/ml of Con A. These antigen or mitogen concentrations resulted in optimal stimulatory effects in preliminary experiments (17). After 48 h of culture, the wells were emptied. Secreted and bound IFN-γ was visualized by sequential application of polyclonal rabbit anti-rat IFN-γ (Innogenetics), biotinylated anti-rabbit IgG and avidin–biotin peroxidase complex (ABC) (both from Dakopatts, Glostrup, Denmark). After peroxidase staining, the red–brown immunospots, which corresponded to the cells that had secreted IFN-γ, were enumerated in a dissection microscope. To calculate the numbers of T cells responding to a particular antigen or mitogen, numbers of spots in culture without antigen added were subtracted from the values obtained after antigen or mitogen exposure. The data were expressed as numbers/10^5 MNC.

Table 1. Sequences of the primers used for RT-PCR amplification

Cytokine	Accession no.	Primer sequences	Product size (bp)
IFN-γ	K00083	5′-TCAGCAACAACATAAGCGTC-3′ 5′-GAATCAGCAGCGACTCCTTT-3′	212
IL-4	M25892	5′-CGGCATTTTGAACGAGGTCA-3′ 5′-TGCTCTTTAGGCTTTCCAGG-3′	298
IL-10	M8340	5′-ATAACTGCACCCACTTCCCA-3′ 5′-TCATGGCCTTGTAGACACCT-3′-	393
β-actin	X03672	5′-TGAAGATCCTGACCGAGCGT-3′ 5′-GACTCATCGTACTCCTGCTT-3′	520

Detection of cytokine mRNA expression by semi-quantitative RT-PCR

PILN MNC suspensions of 2×10^6 cells/ml consisting of pools of equal amount of cells from five mice in each group, sacrificed on day 7 post-primary infection (p.i.), were cultured for 24 h with or without AChR (final concentration 10 μl/ml). The cells were washed, divided into aliquots of 5×10^6 cells and stored at –70°C until use. mRNA was extracted from 5×10^6 MNC using a QuickPrep Micro mRNA purification kit (Pharmacia Biotech, Uppsala, Sweden). cDNA was synthesized using oligo$(dT)_{18}$ primer according to the manufacturer's instructions (Pharmacia Biotech). PCR primers for mouse cytokines (IFN-γ, IL-4 and IL-10) were designed by using Oligo 5.0 software (NBI, Plymouth, MN). The sequences of primers used are shown in Table 1. A mouse β-actin gene was simultaneously amplified as a reference gene for normalization. All the primers used were fluorescence labeled. PCR amplification was performed in 10 μl of reaction volumes consisting of 50 mM KCl, 10 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 μM primers, 1 μl cDNA and 0.25 U Taq polymerase (Pharmacia Biotech). Thirty amplification cycles were carried out by the GeneAmp PCR System 9600 (Perkin-Elmer, Foster City, CA), and each cycle consisted of 94°C 30 s, 55°C 30 s and 72°C 30 s, whereas annealing temperature for β-actin was 62°C. Amplified PCR products were pooled with the size standard (GenScan500; Perkin-Elmer) and analyzed on an automated DNA sequencer (ABI-377; Perkin-Elmer). The specific fluorescence intensity

Table 2. B cell-deficient mice are resistant to AChR-induced EAMG

Phenotype	No. of mice per group	Muscle weakness (grade)[a]				No. of mice which died due to severe disease	Disease incidence	P[b]
		0	1	2	3			
Experiment 1								
C57BL/6	17	5	5	3	2	2	12/17 (71%)	
μMT	15	13[c]	0	0	0	0	0/15 (0%)	<0.001
Experiment 2								
C57BL/6	5	1	1	3	0	0	4/5 (80%)	
μMT	6	6	0	0	0	0	0/6 (0%)	<0.01

[a]Mice were immunized repeatedly with AChR + CFA, and monitored for EAMG (muscle weakness graded from 0 to 3 as described in Methods) for 100 days after primary immunization.
[b]Fisher's test.
[c]Two mice died of anesthesia.

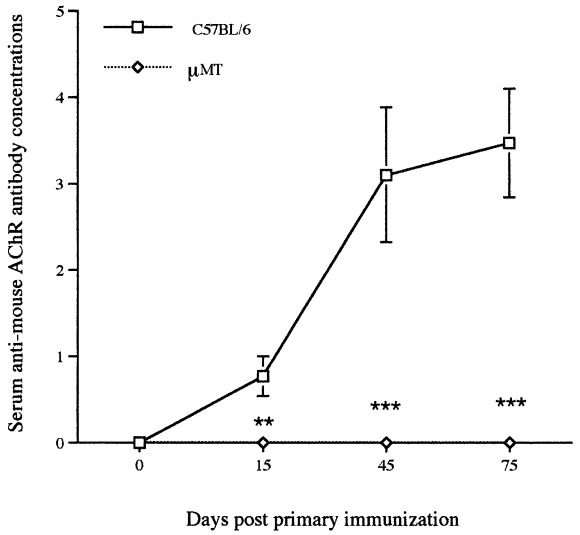

Fig. 1. Anti-AChR antibody levels are undetectable in serum from μMT mice. The antibody concentrations were measured by radioimmunoassay and expressed as moles of α-bungarotoxin binding sites. The results are the mean values of 12 mice per group. Bars refer to SD. $^{**}P < 0.01$, $^{***}P < 0.001$.

determined as peak areas by the sequencer parallels with copies of amplified products. Semi-quantitation of cytokine gene expression was represented as ratios of peak areas of cytokines and those of β-actin.

Enumeration of anti-AChR IgG antibody secreting cells

An ELISPOT assay was used with some modifications (19). Briefly, wells of microtiter plates with nitrocellulose bottoms were coated with 100 μl AChR or MBP (10 μg/ml in PBS). Aliquots of 100 μl cell suspensions containing 2×10^5 MNC were added in triplicate to individual wells. After incubation for 24 h, the wells were emptied, followed by addition of rabbit anti-mouse IgG (Sigma), biotinylated swine anti-rabbit IgG (Dakopatts) and ABC. After peroxidase staining, the red–brown immunospots which corresponded to cells having secreted anti-AChR IgG were counted and numbers were standardized to numbers/10^5 MNC.

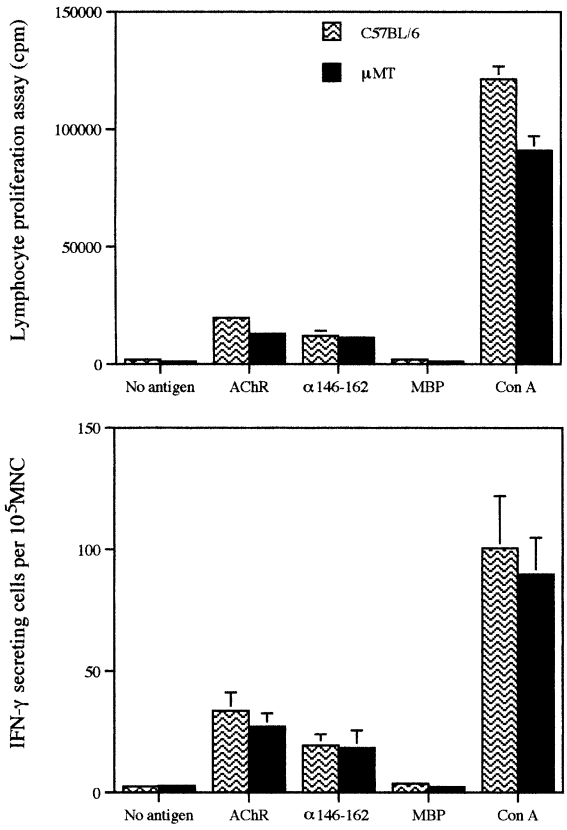

Fig. 2. T cell responses to AChR and its dominant peptide α146–162. Mice were immunized with 20 μg AChR in CFA into footpads and thigh regions. After 7 days p.i., the mice were sacrificed and MNC were isolated from popliteal and inguinal lymph nodes. The results of lymphocyte proliferation are expressed as c.p.m. and those of IFN-γ ELISPOT assays as numbers of IFN-γ-secreting cells/10^5 MNC. Symbols refer to mean values of four mice per group and bars to SD. $^{**}P < 0.01$, $^{***}P < 0.001$. The results presented are representative of three independent experiments.

Radioimmunoassay for measurements of serum anti-AChR antibodies

Blood samples were collected from mouse tail vein just prior to immunization, and then on day 15, 45 and 75 after primary

Table 3. Cytokine mRNA expression in MNC cultured with and without AChR

Phenotype	IFN-γ		IL-4		IL-10	
	–[a]	AChR	–	AChR	–	AChR
C57BL/6	0.02[b]	0.66	0.01	0.03	0.05	0.49
μMT	0.02	0.61	0.01	0.04	0.03	0.40

[a]No antigen stimulation.
[b]Cytokines were detected by a fluorescence-based semi-quantitative RT-PCR method. Cytokine transcriptional levels were expressed as ratio of fluorescence peak areas of cytokines and those of β-actin (1:10 dilution) detected by an ABI 377 DNA sequencer.

immunization. Serum anti-AChR antibody concentrations were measured by radioimmunoassay (14). Briefly, 1 nM M-AChR was incubated with 2 nM [^{125}I]α-bungarotoxin (Amersham). To 1 ml of labeled M-AChR, 1 μl serum was added, followed by rabbit anti-mouse Ig (Dakopatts). The samples were centrifuged, washed and counted in a γ-counter. The AChR precipitated minus the background value permits calculation of the titer in moles of toxin binding sites bound per liter of serum.

Immunohistochemistry

Immunohistochemistry studies were performed on groups of 4 mice on day 15, 45 and 75 p.i. (20). Cellular infiltrates were characterized in serial sections by incubation with the following mAb: rat anti-mouse CD4 and CD8 (PharMingen, San Diego, CA), and rat anti-mouse Mac-1 (Boehringer, Mannheim, Germany), followed by biotinylated rabbit anti-rat IgG (Vector, Burlingame, CA) and ABC (Vector). The numbers of macrophages, CD4$^+$ and CD8$^+$ cells were counted in transverse sections of five random fields of 1 mm^2 at a magnification of ×200 per marker and animal. Data from three sections of each muscle were pooled. The mean values for both limbs provide a mean value for each animal. The results were expressed per 1 mm^2 tissue area. For co-localization of cellular infiltration and AChR, the same sections were incubated with FITC-labeled α-bungarotoxin (Sigma).

Statistical analysis

Differences between the two groups were evaluated by Student's *t*-test. Differences between groups with respect to the incidence were analyzed with Fisher's exact test.

Results

B cell-deficient mice are resistant to EAMG induction

In the first experiment, 15 μMT and 17 B6 mice were immunized 3 times (days 0, 30 and 60) with AChR in CFA. Twelve B6 mice developed muscle weakness before (two mice) or after (10 mice) the first boosting. Among them, five mice exhibited mild or moderate muscle weakness. Seven mice deteriorated progressively and two of them died, while one was killed humanely for animal protection reasons. In contrast, μMT mice remained completely resistant to EAMG induction. Only one out of 17 exhibited mild weakness and recovered around day 54 p.i. Two μMT mice died from anesthesia during the experiments. In a second experiment series with five B6 and six μMT mice, similar results were obtained. Four B6 mice but none of the μMT mice developed clinical muscle weakness (Table 2).

AChR-specific IgG antibody-secreting cells and serum anti-AChR antibodies were not detected in μMT mice

The B6 mice immunized with AChR + CFA had high numbers of anti-AChR IgG antibody secreting cells when examined on day 7 p.i. (mean 45 ± 12/10^5 MNC). Such cells were not detectable in the immunized μMT mice. Consistently, levels of serum anti- AChR antibodies increased gradually in the B6 mouse, but were undetectable in the μMT mice (Fig. 1). Numbers of MNC secreting IgG antibodies to the control antigen MBP were 0–1.2 cells/10^5 MNC in B6 mice and also absent in μMT mice.

Lymphocyte proliferation and IFN-γ-secreting cells were not affected μMT mice

We immunized μMT and wild-type B6 mice with 20 μg of AChR in CFA. Seven days later, T cell responses of draining lymph nodes were analyzed *in vitro* for proliferation and levels of IFN-γ-secreting cells in response to AChR and the α146–162 peptide. As shown in Fig. 2, MNC from μMT and wild-type B6 mice responded similarly to AChR and α146–162. Thus, AChR and peptide-specific T cell priming remained unaffected in μMT mice.

μMT and wild-type B6 mice have similar levels AChR-reactive cytokine mRNA

To further define T cell functions in μMT mice, we examined cytokine mRNA levels in draining lymph node MNC by semiquantitative RT-PCR, which was shown in preliminary experiments to have high sensitivity and specificity (data not shown). Upon stimulation with AChR, μMT and control B6 mice had similar mRNA levels of IFN-γ, IL-4 and IL-10 (Table 3). The mRNA levels detected after culture without AChR were low. No difference was found for mRNA levels of these cytokines between the two groups (Table 3).

Muscular infiltrations

After immunization with AChR in CFA, macrophages, CD4$^+$ and CD8$^+$ cells, can be detected at day 15, 45 and 75 p.i. in muscle sections of control B6 and μMT mice (Fig. 3). There was no obvious fluctuation in numbers of infiltrating cells at the three time points. There was also no difference in numbers of infiltrating cells between μMT and control B6 mice. The mean numbers were 31 ± 11 cells/mm^2 for macrophages, 17 ± 10 cells/mm^2 for CD4$^+$ cells and 3 ± 1 cells/mm^2 for CD8$^+$ cells at the three time points in the μMT mice, and 27 ± 14 cells/mm^2 for macrophages, 20 ± 6 cells/mm^2 for CD4$^+$ cells and 4 ± 2 cells/mm^2 for CD8$^+$ cells in the control B6 mice. Macrophages and CD4$^+$ cells, but not CD8$^+$ cells could be detected in muscle of CFA immunized mice (mean 2 ± 2 cells/mm^2) (Fig. 3a). Most infiltrating CD4$^+$ cells and macrophages were localized in the vicinity of neuromuscular junctions (Fig. 3d).

Discussion

We examined the role of B cells in the development of EAMG. μMT mice are completely resistant to EAMG induction,

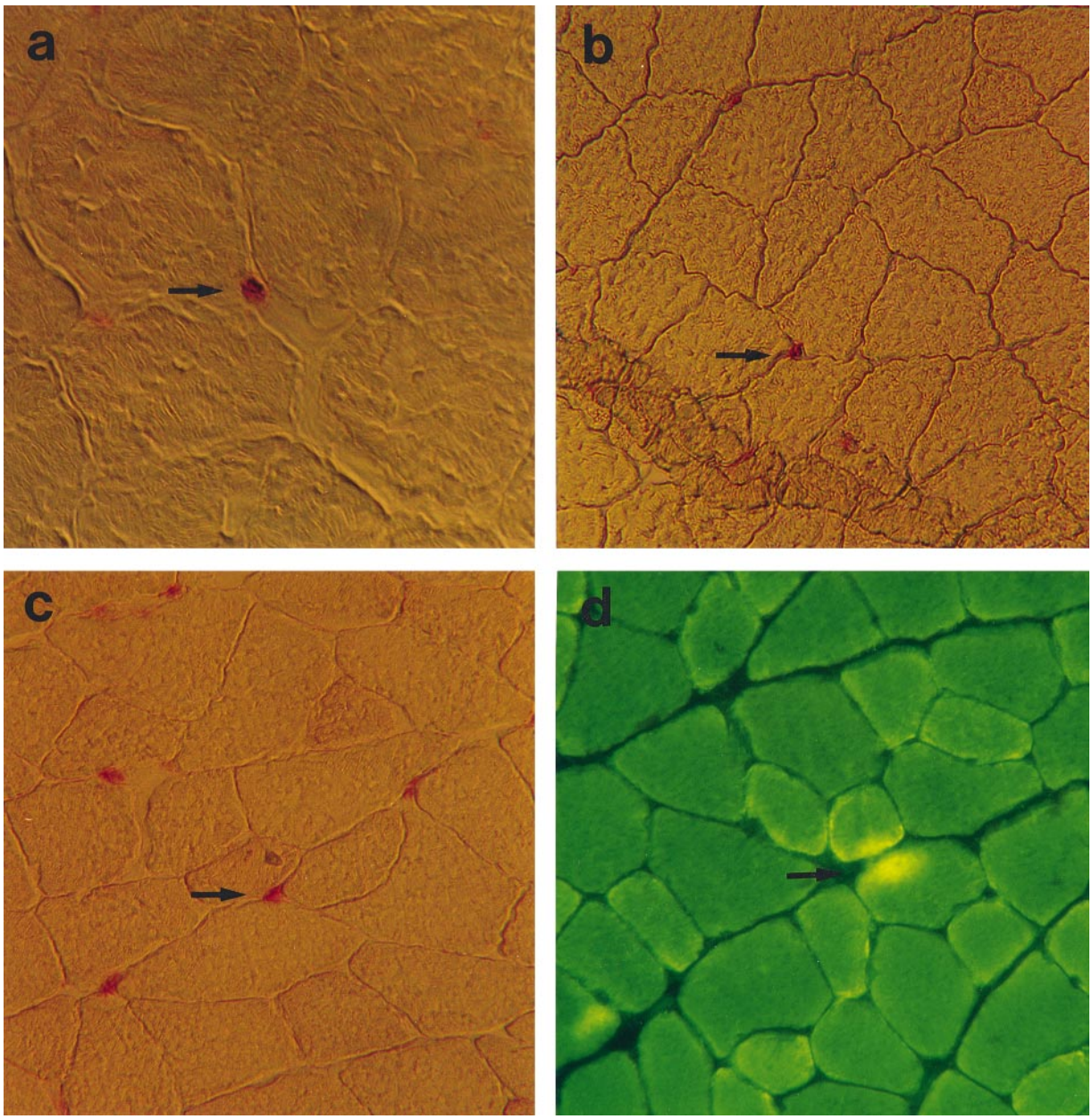

Fig. 3. Immunohistochemistry analysis of striated muscle sections from C57BL/6 (B6) μMT and wild-type B6 mice p.i. with AChR in CFA, or CFA alone. (a) Very few macrophages are seen in muscle sections from a B6 mouse on day 15 p.i. with CFA only. (b) $CD4^+$ T cells from a μMT mouse on day 45 p.i. with AChR + CFA. Arrow denotes positive cells scattered in the perimysium. (c). Macrophages (arrows) from a μMT mouse on day 15 p.i. with AChR + CFA. (d). The same sections were stained for AChR (arrows) with FITC labeled α-bungarotoxin. Magnifications: a, ×400; b–d, ×200.

associated with no detectable anti-AChR antibody production, but normal T_h functions. These results unequivocally demonstrated the absolute requirement of B cells and pathogenic anti-AChR antibodies in the genesis of EAMG, while the role of additional effector mechanisms may only act in cooperating B cells/antibodies in EAMG pathogenesis.

AChR-targeted neuromuscular interference can result from several autoantibody-mediated effects, including functional blockade, AChR cross-linking and complement activation (5). Because of the poor correlation between antibody levels and severity of clinical disease, and the presence of inflammatory cells in muscle specimens in both MG and EAMG, it is not clear whether there exists a role of cellular immunity in MG pathogenesis. Further, depletion of $CD8^+$ T cells either by antibody or by gene knockout reduces the clinical severity of EAMG in Lewis rat and B6 mice (21,22). $CD8^+$ T cells are suggested to be involved as helper or effector cells in EAMG pathogenesis (21,22).

In human MG, anti-AChR antibody levels in different patients do not correlate with the severity of clinical muscle weakness,

changes of the antibody levels in a given patient generally correlate well with changes in clinical disease severity (23). Seronegative MG patients clearly have an antibody-mediated disorder since their serum IgG can passively transfer neuromuscular block to naïve animals and seronegative MG patients benefit from plasma exchange (3). It has become more and more evident that certain anti-AChR antibodies with high disease-causing potential, even when present at extremely low levels, can be highly detrimental to the functioning of the neuromuscular junction (5,24–26). Antibody-binding affinity, fine specificity for AChR epitopes and antibody isotypes determine the outcome of disease (5,17,25,26).

Infiltrating macrophages may contribute to muscle weakness by complement-dependent cell-mediated cytotoxicity in the early phase in EAMG in Lewis rat (8,9). However, this early phase of muscle weakness cannot be recorded in human MG and EAMG in mouse. Accordingly, only the chronic phase of EAMG in Lewis rats appears to represent an antibody-mediated event (27). A major proportion of infiltrating macrophages is eliminated by apoptosis soon after their appearance (28). Macrophage-associated cytokines but no chemokine mRNA-expressing cells could transiently be detected in muscle, which might indicate that the immune effector responses in muscle tissue in EAMG are not operated by infiltrating cells (20). The paucity of T cells at or near the end-plates suggests that antigen presentation to T cells may not occur locally. A recent study, in support of this, showed that macrophages do not contribute to AChR loss in the effector phase of passive transfer EAMG (29). Unlike in Lewis rat EAMG, we showed that muscle infiltrations of macrophages, $CD4^+$ and $CD8^+$ T cells are constantly low, and there is no difference in numbers of infiltrating cells in immunized wild-type B6 and μMT mice. The expansion and cytotoxic T lymphocyte activity have been shown not affected in the absence of B cell (30). In MG patients, inflammatory infiltrations are not topographically related to end-plates and are not associated with severity of disease (6). Taken together, the infiltrating cells in muscle are unlikely to have any pathogenic role in EAMG or MG. The reduced clinical severity of EAMG after $CD8^+$ T cell depletion or knockout may be the result of lacking the regulatory role of $CD8^+$ T cells.

The production of anti-AChR antibody is dependent on T cell help, provided by T_h1 and T_h2 cytokines and on cross-linking with the CD40 ligand (19). In the present study, T cells proliferated normally to AChR and its myasthenogenic peptide α146–162 in μMT and control B6 mice, and both strains expressed similar levels of IFN-γ, IL-4 and IL-10. Therefore, B cells seem not to be required for priming naïve T cells *in vivo*. Our data support the hypothesis that the APC initiating in immune response to peptide antigen is most likely the dendritic cells, as suggested from recent studies (31–33). T cell priming remained intact in this setting, suggesting that the resistance to EAMG induction in μMT mice is not the result of impaired T_h functions.

μMT mice develop autoimmune encephalomyelitis and diabetes at a similar magnitude as wild-type mice (31,33). In contrast to these T cell-mediated conditions, μMT mice become completely resistant to EAMG induction. In accordance with this finding, we have also shown that CD40 ligand-deficient mice had greatly impaired antibody responses and hence are resistant to EAMG induction (19). Thus, the present studies reinforce the notion that B cells and autoantibodies are critical in EAMG pathogenesis and perhaps in human MG as well.

Acknowledgements

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Abbreviations

APC	antigen-presenting cell
AChR	acetylcholine receptor
CFA	complete Freund's adjuvant
Con A	concanavalin A
EAMG	experimental autoimmune myasthenia gravis
μMT	B cell knockout
MBP	myelin basic protein
MNC	mononuclear cell
p.i.	post-primary immunization
PILN	popliteal and inguinal lymph nodes

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