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Split Tolerance in a Novel Transgenic Model of Autoimmune Myasthenia Gravis

Sue Stacy,* Bruce E. Gelb,* Barbara A. Koop,2§ Jolene J. Windle,3§ Katherine A. Wall,¶ Keith A. Krolick,‡ Anthony J. Infante,‡ and Ellen Kraig*4

Because it is one of the few autoimmune disorders in which the target autoantigen has been definitively identified, myasthenia gravis (MG) provides a unique opportunity for testing basic concepts of immune tolerance. In most MG patients, Abs against the acetylcholine receptors (AChR) at the neuromuscular junction can be readily identified and have been directly shown to cause muscle weakness. T cells have also been implicated and appear to play a role in regulating the pathogenic B cells. A murine MG model, generated by immunizing mice with heterologous AChR from the electric fish Torpedo californica, has been used extensively. In these animals, Abs cross-react with murine AChR; however, the T cells do not. Thus, to study tolerance to AChR, a transgenic mouse model was generated in which the immunodominant Torpedo AChR (T-AChR) α-subunit is expressed in appropriate tissues. Upon immunization, these mice showed greatly reduced T cell responses to T-AChR and the immunodominant α-chain peptide. Limiting dilution assays suggest the likely mechanism of tolerance is deletion or anergy. Despite this tolerance, immunization with intact T-AChR induced anti-AChR Abs, including Abs against the α-subunit, and the incidence of MG-like symptoms was similar to that of wild-type animals. Furthermore, evidence suggests that this B cell response to the α-chain receives help from T cells directed against the other AChR polypeptides (β, γ, or δ). This model offers a novel opportunity to elucidate mechanisms of tolerance regulation to muscle AChR and to clarify the role of T cells in MG. The Journal of Immunology, 2002, 169: 6570–6579.

Numerous transgenic mouse systems have been developed to study immune tolerance to self-Ags. Yet in many of these models, the Ag that is implicated in the human autoimmune disorder has not been used, often because the disease-causing Ag had not been identified (1). Thus, we have chosen to focus on myasthenia gravis (MG), one of the few autoimmune disorders in which the self-Ag has been clearly delineated. MG is mediated by Abs against the patient’s acetylcholine receptors (AChR; primarily the α-chain) at the neuromuscular junction (2).

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5 Abbreviations used in this paper: MG, myasthenia gravis; AChR, acetylcholine receptor; T-AChR, Torpedo AChR; EAMG, experimental autoimmune MG; HPRT, hypoxanthine phosphoribosyl transferase; LNC, lymph node cell.

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lysine at p155. Therefore, the primary T cell response is directed at T-AChR with no recognition of the mouse AChR; it is not a true “autoimmune” response. Only T cells with “self”-reactivity are subject to tolerance; therefore, the mechanisms of tolerance generation, maintenance, and circumvention cannot be addressed.

Thus, a new MG model has been developed by deriving a line of transgenic mice in which the T-AChR α-chain is expressed as a “neo” self-Ag. This is analogous to other systems in which immune tolerance has been generated against products of transgenes (27–31). For example, studies of mice expressing either a viral glycoprotein or β-galactosidase in the thymus both suggest that higher levels of expression will result in more complete central tolerance (29, 30). In another study, mice were generated in which hen egg lysozyme was expressed in different tissues; the degree of peripheral tolerance induced depended on the site of protein expression (31). In general, the degree and mechanism of tolerance varies with level, location, and timing of expression of the transgene, as well as with factors affecting the avidity of the TCR interaction with the peptide-MHC complex (29–32). Importantly, the T-AChR α-chain transgenic model offers the first opportunity to assess tolerance to the muscle AChR, the immunogenic protein in MG.

Materials and Methods

Generation of the T-AChR α-chain transgenic mouse

The transgenic construct is shown diagrammatically in Fig. 1. Briefly, it was generated using the vector pBSpKCR3ΔRI, which provides a cleavable intron and a polyadenylation site derived from the rabbit β-globin gene (33). There are no methionine codons within the globin sequences upstream of the EcoRI cloning site; therefore, translation will initiate at the appropriate ATG within the 1760-bp AchRα cDNA sequence inserted. The cDNA used, from pSS2-T-AChRα, was cloned and kindly provided by Dr. T. Claudio (34). The promoter incorporated was a 850-bp BamHI fragment from upstream of the chicken AChR α-chain gene; it was subcloned from plasmid pSK-Chω, which had been provided by Dr. J. Merlie (35). This promoter had been previously shown to direct transcription of a reporter gene to muscle tissue primarily at the neuromuscular junction (35, 36). The transgenic construct was excised from the plasmid by XhoI digestion and used to produce transgenic mice (37). The presence of the transgene was identified in resulting offspring by Southern blot analysis of tail tissue DNA hybridized with the 32P-labeled XhoI fragment. Transgenic mice of subsequent generations were identified by PCR with primer sets that could distinguish between the I-Aα2 and the I-Aα3 alleles (Table I). In each backcross generation, offspring were selected that were homozygous for I-Aα2 and carried the T-AChR transgene. The transgenic mice were housed in a specific pathogen-free animal facility and used in protocols approved by our institution.

Expression of the T-AChR α transgene

RNA was isolated from murine tissues by homogenization in guanidinium thiocyanate and centrifugation on a cesium chloride gradient (38). The poly(A)-containing mRNA was enriched by passage over an oligo(dT)–cellulose column (Collaborative Research, Lexington, MA). CDNA was generated from 650 ng of each mRNA by incubation with 1 μl of oligo(dT) primer (Pharmacia, Piscataway, NJ) and 200 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, MD) under standard conditions (39) in a 100-μl reaction. The CDNA was extracted with phenol and chloroform, ethanol precipitated, and resuspended in 50 μl of TE (pH 7.5). PCR was performed using 1 μl of each cDNA in a 50-μl reaction; the primer set (CK-HM/TR3) and PCR conditions are summarized in Table I. Where indicated, samples were taken in duplicate from the PCR at three-cycle intervals, electrophoresed on a 2% agarose gel, stained with ethidium bromide, and visualized with an AlphaImager (Alpha Innotech, San Leandro, CA). To increase sensitivity, the PCR products were transferred to a Duralon-UV membrane (Stratagene, La Jolla, CA) and the resulting Southern blot was hybridized at 42°C with an internal 32P-labeled oligonucleotide probe (5′-TAGACCAACCCCTGATGCCC-3′). After hybridization, the blots were washed in 2× SET buffer (300 mM sodium chloride, 2 mM EDTA, and 60 mM Tris) with 0.01% SDS at 42°C, and the radioactivity was quantitated directly using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). As a normaliza-
tion control, CDNA samples were amplified in parallel using primers across an intron within the hypoxanthine phosphoribosyl transferase (HPRT) gene and subsequently hybridized at 42°C with an internal probe (5′-GGATATGCCCTTGACTAATGG-3′). Using values within the linear range of the PCR for each tissue, the 32P pm of the T-AChR PCR products were normalized to the HPRT signals.

To compare the levels of muscle expression of the T-AChR α-chain with the endogenous mouse AChR α-chain, RT-PCR for increasing numbers of cycles was conducted with primers specific for either the murine AChR α chain (murine sequences (5′-ATCGACAAAACTGATGCCC-3′). After hybridization, the blots were washed in 2× SET buffer (300 mM sodium chloride, 2 mM EDTA, and 60 mM Tris) with 0.01% SDS at 42°C, and the radioactivity was quantitated directly using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). As a normali-
zation control, CDNA samples were amplified in parallel using primers across an intron within the hypoxanthine phosphoribosyl transferase (HPRT) gene and subsequently hybridized at 42°C with an internal probe (5′-GGATATGCCCTTGACTAATGG-3′). Using values within the linear range of the PCR for each tissue, the 32P pm of the T-AChR PCR products were normalized to the HPRT signals.

Immunizations

AChR was obtained from the electric ray, T. californica (Pacific Bioma-
rine, Venice, CA) by affinity chromatography on cobra toxin conjugated to Sepharose 4B (40). To facilitate the study of tolerance to the α-chain alone, a T-AChR α-chain fragment (aa 1–210) was generated from the original cDNA by PCR using the TAcHR-RES1 primers (Table I). The PCR product was cloned into pRSET A (Invitrogen, Carlsbad, CA) and expressed in Escherichia coli (BL21 pLysS). The recombinant protein had a histidine tag at its amino terminus which facilitated purification over a Ni-NTA column (Qiagen, Valencia, CA). The purified AChR α-chain fragment was then refolded using an oxidido-shuffling method, as described by others (41, 42). Briefly, the protein was treated with DTT to reduce disulfide bonds and then incubated in a renaturation buffer containing oxidized and reduced forms of glutathione, t-arginine, EDTA, and Tris-HCl (42). Following dialysis, the protein was concentrated using Amicon Centriplus YM-10 units (Millipore, Bedford, MA).

The intact T-AChR, the p146–162 peptide (Protein Chemistry Core Fa-
ility, Baylor College of Medicine, Houston, TX), and the recombinant T-AChR α-chain fragment (aa 1–210) were used for injections, as indicated in the text.

For T cell experiments, mice were immunized s.c. at the base of the tail with 50 μl of emulsion containing either 25 μg of T-AChR or 1 μg of p146–162 peptide and 25 μl of CFA. For Ab studies, mice were immu-
nized s.c. at live sites (above each shoulder, above each thigh, and at the base of the tail) with a total of 100 μl of emulsion containing 50 μg of...
T-AChR and 50 μl of CFA or, in one study, with 10 μg of T-AChR α-chain fragment (α1–210) in CFA.

For analysis of disease susceptibility, mice were given T-AChR and a simultaneous course of IL-12 (kindly provided by Genetics Institute, Cambridge, MA) (43). The IL-12 was dissolved in PBS with 1% normal mouse sera, and beginning 1 day before T-AChR immunization, 1 μg was given i.p. daily for 5 days. The mice were immunized s.c. at the base of the tail with 25 μg of T-AChR or with PBS in an equal volume of CFA. In a secondary immunization, given 1 mo later, the mice received the same IL-12 regimen with T-AChR/CFA or PBS/CFA. For subsequent immunizations, no IL-12 was given. For both secondary and tertiary exposures, the Ag was given s.c. in multiple sites.

T cell assays

T cell proliferation assays were performed as described previously (23). Briefly, 7 days after immunization, inguinal and paraaortic lymph nodes were removed and single-cell suspensions were prepared by mechanical disruption in RPMI 1640 supplemented with 10% FBS, 10 mM HEPES, 2 mM L-glutamine, 50 μM 2-ME, 50 U/ml penicillin, and 50 μg/ml streptomycin. The lymph node cells (LNC; 2.5 × 10^6 cells/well) were cultured in flat-bottom 96-well plates (Falcon; Corning Glass, Corning, NY) with varying concentrations of T-AChR or peptides of the T-AChR α-chain. The peptides p111–198, p146–210, p182–198, and p360–378 were prepared as described elsewhere (7). The plates were incubated for 96 h, pulsed with 1 μCi of [3H]thymidine/well, and harvested 18 h later. [3H]Thymidine uptake was measured by liquid scintillation spectrometry and the mean cpm of triplicate wells ± SEM was calculated after subtraction of background.

The frequencies of T cells specific for p146–162 were assessed by limiting dilution assays as previously described (44). In brief, LNC were plated at concentrations from 0.6 to 10 × 10^4 cells/well. Spleen cells from C57BL/6 mice were irradiated (3000 rad) and pulsed with 10 μM T-AChR p146–162 for 18 h and were then added (1 × 10^5 cells/well) as APC. Additional irradiated spleen cells were added to maintain a consistent 2 × 10^5 cells/well. The T cell response was measured using uptake of [3H]thymidine as described above. Those wells in which the [3H] cpm exceeded background by 1 SD were considered positive (45). The fraction of negative wells was plotted against the total number of cells plated per well, and the Poisson statistic was used to derive the precursor frequency.

Measuring levels of anti-T-AChR Abs

The titers of serum anti-AChR Abs were measured by standard ELISA as previously described (46). Briefly, 96-well ELISA plates (Titertek, Cleveland, OH) were coated with 50 μg/ml T-AChR (20 μg/ml in PBS) for 2 h at room temperature. After washing the wells with 0.05% Tween 20 in PBS, the plates were blocked with 200 μl/well 1% BSA in PBS for 1 h at room temperature and kept overnight at 4°C. After washing, 50 μl of each test mouse serum, diluted in 1% BSA/PBS, was added. Five-fold dilutions from 1/25 to 1/78,125 were tested in duplicate. Plates were incubated at room temperature for 2 h, washed, and incubated for 1 h with 50 μl/well rabbit anti-mouse IgG (whole molecule) conjugated to HRP (Sigma-Aldrich, St. Louis, MO). After washing as above, 100 μl of ABTS with 0.03% hydrogen peroxide was added per well and developed for 10–30 min. Absorbance at 410 nm was read on a Dynatech MRX ELISA platereader (Dynatech Laboratories, Chantilly, VA). Titers were plotted graphically and the average OD ± SEM was plotted for each dilution point. The positive control was a serum with known reactivity to T-AChR, and the negative controls used were 1% BSA in PBS and sera from unimmunized transgenic and nontransgenic mice.

### Table 1. PCR primers used

<table>
<thead>
<tr>
<th>Primer</th>
<th>Specificity</th>
<th>Sequence (5’-3’)</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK-HM/TR3</td>
<td>Transgene</td>
<td>GCTGAGAGGAGCOGCCGCT/</td>
<td>80°C, 2 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GTGGGTGTGTAGCTCCACTG</td>
<td>95°C, 1 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>55°C, 1 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>72°C, 1 min</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>≤34 cycles</td>
</tr>
<tr>
<td>TR1/TR2</td>
<td>T-AChRα</td>
<td>CGTCCAGTGGACATCACACCCAC/</td>
<td>80°C, 2 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GATATCCAGGTAAGGAAGTGCAGG</td>
<td>95°C, 1 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>55°C, 1 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>72°C, 1 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>30 cycles</td>
</tr>
<tr>
<td>MR1/MR2</td>
<td>Mouse AChRα</td>
<td>CGGCCAGTGGACCACACGGTGA/</td>
<td>94°C, 2 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GATGTCAGGGGAGGATGTGGGG</td>
<td>94°C, 1 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>55°C, 1 min</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>≤35 cycles</td>
</tr>
<tr>
<td>I-AAb1/I-AAb2</td>
<td>I-Aαβ</td>
<td>CACCTATGGTATAAGTGA/</td>
<td>80°C, 2 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGGGTCAAACGTCCCAA</td>
<td>95°C, 1 min</td>
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<td></td>
<td>42°C, 1 min</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>72°C, 1 min</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>30 cycles</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 cycle</td>
</tr>
<tr>
<td>I-AAd1/I-AAd2</td>
<td>I-Aαδ</td>
<td>CTTCTATGGTACAAGTGT/</td>
<td>Same as I-Aαβ</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGGCTCAAAGAGTACCAA</td>
<td></td>
</tr>
<tr>
<td>HPRT6/HPRT7</td>
<td>HPRT</td>
<td>GTTGGATACAGGCCAGCTGGTG/</td>
<td>80°C, 2 min</td>
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<tr>
<td></td>
<td></td>
<td>GATTCGACTTGCTGCTACCTAGGC</td>
<td>95°C, 1 min</td>
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<td></td>
<td>65°C, 1 min</td>
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<td></td>
<td>72°C, 1 min</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>≤27 cycles</td>
</tr>
<tr>
<td>TACHR-RSET 5’/</td>
<td>α1-210</td>
<td>ATAGAGCTCTCTGAACATGAACACAGTTGG/</td>
<td>85°C, 1 min</td>
</tr>
<tr>
<td>TACHR-RSET 3’</td>
<td></td>
<td>ATAGGTACCTTAATACGCTGCAATGATAAAAATGG</td>
<td>10°C, 1 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>50°C, 30 s</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>72°C, 1 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>25 cycles</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 cycle</td>
</tr>
</tbody>
</table>
Measuring Ab responses to individual T-AChR chains

Purified T-AChR was reduced and denatured by boiling in SDS loading buffer with 5% 2-ME. The receptor was then applied to a 9% SDS-polyacrylamide gel and electrophoresed at 30–35 mA for several hours to separate the individual polypeptide chains (47). The gel was negatively stained using Copper stain (Bio-Rad, Hercules, CA), and the bands were excised. After destaining with Tris/glycine solution (Bio-Rad), the acrylamide piece was dispersed by forcing it through a 25-gauge needle. Protein was then eluted from the crushed gel by incubating the samples at 37°C for 30 min in Tris-EDTA buffer (pH8) with 1% 2-ME and 0.1% SDS. Eluted protein from each chain was loaded into separate wells and electrophoresed on a 9% SDS-polyacrylamide gel. The fractionated proteins were electroblotted to an Immobilon-P membrane (Millipore), which was then blocked with 5% BSA in TBS, washed, and incubated with primary Ab (1/200 dilution). After washing, the blots were incubated with goat anti-mouse (polyvalent; Ab (1/1000 dilution) conjugated to alkaline phosphatase. Color was developed by addition of nitroblue tetrazolium/5-bromo-4-chloro-3-indoylphosphate substrate (Promega).

Assays for myasthenic symptoms

After immunization with T-AChR/CFA and IL-12, two different assays were used to score development of MG. First, a classical clinical score was assigned as previously described (48). Myasthenic symptoms were graded on the following scale: 0, no disease; 1.0, normal at rest but displays myasthenic symptoms during exercise including chin down, flaccid tail, and forelimb weakness; 2.0, weakness as in stage 1 but shown at rest before exercise; 3.0, severe weakness, dehydration, and paralysis of hind limbs; and 4.0, death. As a secondary measure, a grip strength device with a precision force gauge was used (Columbus Instruments, Columbus, OH). Mice were exercised by 12 gentle pulls across the cage grid (backward walking) and were then allowed to grasp the T-bar of the meter which recorded peak force on a digital display. The maximum force of the three pulls was recorded. This was repeated twice for a total of three peak force values, which were then averaged.

Results

Generation of the T-AChR α transgenic mouse model

To create a suitable model for studies of T cell tolerance to the major autoantigen in MG, AChR, a transgenic mouse was generated in which the T-AChR α-chain should be expressed endogenously as a novel self-protein. To ensure correct physiologic expression of the transgene, the promoter for the chicken AChR α-chain gene was used; it had been shown previously to direct transcription of a reporter gene primarily at the neuromuscular junctions in transgenic animals (35). The Torpedo α-chain and the mouse α-chain share about an 80% sequence homology. Moreover, it has been previously shown that the T-AChR α-chain can combine with mammalian β-, γ-, and δ-chains to produce a functional receptor in transfected NIH3T3 cells (49). Therefore, it is likely that the resulting transgenic mouse will express some chimeric receptors comprised of the T-AChR α-chain in association with the murine β, δ, and ε AChR chains. Once a transgenic founder was produced, the T-AChR α transgene was then bred to C57BL/6. Using transgenic and nontransgenic littersmates, ~10 wk of age, RNA was purified from six different tissues and analyzed by RT-PCR using primers designed across the vector-encoded intron (CK-HM/TR3 in Table I). These primers could distinguish between an RT-PCR product derived from RNA (546 bp) and one derived from potentially contaminating DNA (~900 bp). PCR products were analyzed on Southern blots (Fig. 2A) hybridized with internal 32P-labeled oligonucleotide probes and were normalized to HPRT products amplified in parallel. As shown in Fig. 2B, the T-AChR α-chain transgene is expressed predominately in muscle tissue, with ~100-fold lower expression in the brain, and trace levels in thymus. As expected, no transgene-derived RNA was detected in kidney, spleen, or liver (Fig. 2). In addition, when tissues from a 4-wk-old mouse were examined, the same expression profile of the transgene was observed, although thymic expression was slightly higher at this earlier developmental stage.

To assess the relative levels of expression of the T-AChR α transgene and the endogenous mouse AChR α gene in muscle, RT-PCR was performed with primers that distinguish the Torpedo and murine α-chain sequences (TR1/TR2 and MR1/MR2 in Table I). The resulting PCR products were analyzed on Southern blots hybridized with an internal oligonucleotide probe common to both the Torpedo and murine gene sequences. As expected, muscle from a nontransgenic littermate showed expression of only the endogenous mouse AChR (Fig. 3A). On the other hand, muscle from transgenic mice contained both murine and Torpedo AChR mRNAs and they were present at approximately equivalent levels (Fig. 3).

To determine the effect of the expressed T-AChR α-chain on T cell responses, mice were immunized with the immunodominant T-AChR α peptide, p146–162, in CFA at the base of the tail. Seven days later, draining lymph nodes were harvested and tested in vitro for activation. As expected, T cells from nontransgenic mice proliferated vigorously in response to stimulation with either T-AChRα p146–162 (Fig. 4A) or native T-AChR (Fig. 4B). On the other hand, lymph node T cells from the transgenic mice showed markedly reduced proliferation in response to stimulation with p146–162 (Fig. 4A). Moreover, when primed in vivo with p146–162, the in vitro response to the entire multichain T-AChR complex was clearly diminished (Fig. 4B). Thus, expression of the T-AChR α-chain in these transgenic mice appears to induce some level of immune tolerance in the reactive T cells.

The diminished reactivity to p146–162 in lymph node T cells from transgenic mice could be due to either a reduction in the magnitude of the response from individual T cells or to a reduction...
in the overall number of responding cells. To address this issue, limiting dilution analysis was performed. Transgenic and nontransgenic mice were immunized with T-AChR/CFA; LNC were harvested 7 days later and assayed for reactivity with p146–162-primed Ag APC, as described in Materials and Methods. The LNC from the two nontransgenic mice demonstrated very similar frequencies of p146–162-reactive cells; the number of T cells responding to p146–162 was ~1 in 12,500 (Fig. 5). On the other hand, in the two transgenic mice, there were many fewer responding T cells; 1 in 78,000 T cells proliferated in vitro to p146–162. Thus, tolerance to p146–162 of the T-AChR α-chain is apparently due to an overall reduction in the number of responding T cells.

**T cell response to intact T-AChR**

Given that T cells specific for the immunodominant peptide p146–162 showed evidence of tolerance in the transgenic mouse, it is possible that other AChR-reactive T cells would be selected (50). To address this, mice were immunized with the intact T-AChR in CFA at the base of the tail. Seven days later, draining lymph nodes were harvested and tested for proliferation in response to in vitro stimulation with either p146–162 or T-AChR. Once again, T cells from the transgenic mice were tolerant to the α-chain peptide (Fig. 6A). However, T cell proliferation was seen in response to T-AChR stimulation (Fig. 6B), presumably due to T cells specific for epitopes on the β-, δ-, and γ-chains. These results were further verified in four additional experiments in which responses from a total of 10 transgenic and 7 nontransgenic mice were examined (data not shown); in every case proliferation to p146–162 was markedly reduced in LNC from the transgenic mice yet a response to intact AChR was clearly evidenced.

To determine whether this reactivity pattern would be altered by multiple immunizations, one nontransgenic mouse and two transgenic mice were immunized three times with 50 μg of T-AChR/CFA. Seven days after the final immunization, LNC were harvested and tested in vitro with four different peptides of the T-AChR α-chain and with the intact T-AChR molecule. As anticipated, primed cells from the nontransgenic mouse proliferated extensively when given the intact T-AChR or the T-AChR α-chain p146–162 peptide in vitro and, to a lesser extent, when given any of the other three α-chain peptides (p111–126, p182–198, p360–378). On the other hand, although LNC from the transgenic mice responded to the intact T-AChR, they showed little measurable response to p146–162 or to any of the other T-AChR α-peptides (Fig. 7). Thus, the T cell tolerance to p146–162 appears to extend to other T cell epitopes on the α-chain and is maintained after multiple exposures to Ag.

**The B cell response after immunization with intact T-AChR**

Typically, mice immunized with T-AChR produce Abs primarily to epitopes on the α-chain. Thus, in our transgenic model, if the B cell response to the α-chain were tolerized (similarly to the T cell response), one might expect an overall decrease in the anti-T-AChR response. Therefore, mice were immunized with T-AChR and the serum titers of Abs specific to T-AChR were measured after 4 wk by standard ELISA. Somewhat surprisingly, there was no difference in the Ab titer to T-AChR seen between the transgenic sera and the sera from nontransgenic mice. This is illustrated by Ab titers from sera taken 4 wk after primary immunization (Fig. 8A); sera taken at other time points in this study showed similar results. Even the use of IL-12 to augment disease development (as described below) did not differentially affect the Ab production by transgenic and nontransgenic mice.
Since only the α-chain of the T-AChR was being expressed in the transgenic mice, we reasoned that this B cell response could be directed toward the other three AChR polypeptides. Thus, to test this hypothesis, the individual chains of T-AChR were separated electrophoretically and were then analyzed on Western blots incubated with sera from the immunized mice. As expected, the sera from nontransgenic mice showed reactivity to both the α-chain and to the other polypeptides as well. The transgenic sera showed a similar pattern (Fig. 8B). Even though the T cell response to the p146–162 peptide from each of the two nontransgenic mice was 1 in 12,500 LNC, whereas the frequency for T cells from each of the two transgenic mice was only ~1 in 78,000.

![FIGURE 5.](image)

The frequency of p146–162-responding T cells in transgenic mice. Frequencies of T cells responding to the p146–162 peptide were determined in limiting dilution cultures in which the number of LNC varied from 0.6 to 10 × 10⁴ cells/well. Response to peptide was measured by a proliferation assay measuring [³H]thymidine incorporation. Positively responding wells were indicated if cpm incorporated exceeded background by 1 SD. The fraction of negative wells was plotted against the total number of LNC plated per well. According to the Poisson statistic, the number of LNC/well that yields a 37% negative culture will contain a single positively responding T cell. The frequency of p146–162-responding T cells from each of the two nontransgenic mice was 1 in 12,500 LNC, whereas the frequency for T cells from each of the two transgenic mice was only ~1 in 78,000.

![FIGURE 6.](image)

Effect of the transgene on the T cell response to T-AChR. Mice were immunized with 25 μg of T-AChR in CFA at the base of the tail. Seven days later, draining LNC were harvested and tested for proliferation to the indicated concentrations of peptide (A) or AChR (B). [³H]Thymidine incorporation was measured by cpm and the in vitro response to peptide was expressed as the difference in the mean cpm with peptide and mean cpm without peptide. All mice were male; #139 and #145 were nontransgenic, while #134 and #142 carried the transgene. Background counts for #139, #145, #134, and #142 were 2163, 1187, 1534, and 525 cpm, respectively.

B cell response after immunization with the recombinant T-AChR α-chain

It is possible that the B cell response to the α-chain in T-AChR-immunized transgenic mice (Fig. 8) receives help from T cells directed against the other AChR chains (β, γ, or δ). To elucidate the role of T cells in this B cell response, mice were immunized with the recombinant T-AChR α-chain fragment, thus eliminating the possibility of T cell help directed at the other AChR polypeptides. The serum titers of Abs specific to T-AChR were measured after 4 wk by standard ELISA. The anti-T-AChR Ab titer of the transgenic mice was clearly reduced over that of the nontransgensics (Fig. 9). Thus, T cell help directed toward other T-AChR chains must have enhanced the anti-α-chain B cell response in the T-AChR-immunized transgenic mice.

mice were analyzed. MG symptoms of grade 2 or higher, with correlating drops in grip strength, were seen in 12 (66%) of 18 of the nontransgenic mice compared with 9 (43%) of 21 of the transgenic mice (Table II). When results were grouped by gender, 7 of 10 females and 5 of 8 males in the nontransgenic group were severely affected, whereas in the transgenic group, 5 of 11 females and 4 of 10 males were clinical stage 2 or higher. The slight decrease in disease incidence seen in the transgenic mice was not statistically significant. Likewise, little differential was seen in disease severity between the transgenic and nontransgenic mice with MG (Table II).
Mice were immunized s.c. at the base of the tail with a total of 100 μl of emulsion containing 50 μg of T-AChR and 50 μl of CFA. Mice were immunized three times, each 1 mo apart. LNC were extracted 7 days after the third immunization and tested for proliferation to a panel of α-chain peptides (0.14 μM each) as well as the intact T-AChR (0.0019 μM). [3H]Thymidine incorporation was measured by cpm and the in vitro response to peptide was expressed as the difference in the mean cpm with peptide and mean cpm without peptide. Background counts for #152, #153, and #113 were 1995, 1329, and 834 cpm, respectively.

**Discussion**

In the transgenic model presented here, the T-AChR α-chain is expressed in muscle tissues at levels essentially equivalent to those of the endogenous α-chain. This “physiological” pattern of expression allows, for the first time, the examination of the mechanisms of T cell tolerance to the AChR α-chain, the target autoantigen in MG. T cell “ignorance” due to the location of the T-AChR α-chain in an immune privileged site can be ruled out in this model since the mice are first primed with the α-chain Ag in vivo and yet remain tolerant as evidenced by the lack of in vitro proliferation in response to α-chain stimulation. Moreover, the low frequency of anti-T-AChR α-chain-reactive T cells in transgenic mice, as determined by limiting dilution assays, suggests that suppression requiring ongoing exposure to a separate cell subset or to a cytokine milieu is not a likely means of tolerance. On the other hand, these studies do not eliminate a possible contribution by suppressive mechanisms; these may have rendered the cells anergic in vivo. Thus, anti-T-AChR α-chain T cells are most probably inactivated either by anergy or deletion in the transgenic mice.

Importantly, trace amounts of the transgene are also found in thymic tissues; this mimics the reported expression of the AChR either by anergy or deletion in the transgenic mice. This bm12 model has a significant Ab response to T-AChR, and tolerance may have been elicited by expression of the transgene, the B cell response to T-AChR is clearly reduced unless the other T-AChR chains are included in the immunogen (Fig. 8). Whether this type of “indirect” T cell help influences the nature or specificity of the B cell response is not yet clear.

Several studies suggest that the nature of T cell help can influence the specificity of the B cell response in the mouse MG model (4, 23, 48). For example, experiments using a murine strain that carries a mutation in the I-Aß (B6.C-H-2bm12) have shown a muted response to p146–162 and a resistance to disease induction (4, 23). Importantly, this bm12 model has a significant Ab response to T-AChR, suggesting a connection between T cell fine specificity and B cell pathogenicity. In this study, we address this issue in a model in which the T-AChR α-chain is now an endogenous Ag...
and the mice are susceptible to development of myasthenic symptoms. This susceptibility to MG in our model presents an interesting contradiction to the lack of disease seen in the bm12 model. One explanation may be that the MHC alterations in the bm12 model affects not only the presentation of p146–162 of the α-chain, but also certain peptides on other AChR chains which might contribute to disease. However, such hypothetical peptides would have to differ enough from α-chain peptides to avoid being tolerogenic in the transgenic model presented here. An alternative explanation is that tolerization of the α-chain in the transgenic model eliminates the activity of suppressor T cells, which could still be at work in the bm12 model.

Interestingly, in the T-AChRα chain transgenic mice, self-directed anti-T-AChR Abs may include not only those against the mouse AChR, but also those that recognize the neo self-T-AChR α-chain determinants. Thus, one might expect disease incidence to be even greater in the transgenic mice than in nontransgenic controls. However, our experiments indicated no increase in MG incidence or severity (Table II) among the transgenic mice; if anything, the induction of myasthenic symptoms occurred with a slightly lower frequency. One explanation is that, as in the bm12 model, when the T cell response to the critical T-AChR α-peptide146–162 is diminished, the proportion of the pathogenic subset of Abs also is altered. This is an intriguing hypothesis since disease severity among MG patients does not correlate directly with overall anti-AChR titer (58). Additional explanations are certainly plausible and, in fact, disease incidence in the transgenic mice (and in MG patients) could be influenced by alterations in other T cell effects, such as cytokine-mediated changes in Ag presentation (59–62).

Thus, it is important that the T-AChRα chain transgenic model presented here allows, for the first time, the opportunity to analyze T cell tolerance to an “endogenous” AChR α-chain in induced autoimmune MG. Autoreactive T cell frequencies, as determined by limiting dilution assays, implicate T cell anergy and/or deletion as the more likely means of tolerization. Furthermore, the incidence of MG development in immunized transgenic mice suggests that alterations in the fine specificity of the T cell response may influence the pathogenicity of the B cell response. Finally, although T cells are clearly implicated in human MG, the extent to which T cell help is provided by leaky tolerance to self-AChR and/or by help from T cells directed against other molecules is not yet clear. This mouse model presents a unique opportunity to 1) assess the contribution of AChR α-chain-specific T cells in providing help to B cells; 2) analyze the effects of T cell specificities on the nature of anti-AChR B cell responses; and 3) implicate the specific mechanisms of tolerance that may be compromised in MG patients.

### Table II. EAMG incidence in transgenic and nontransgenic micea

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<th>Transgenic</th>
<th>Nontransgenic</th>
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<tr>
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<td>No. of mice with MG/total tested</td>
<td>Maximum clinical scores for MG mice</td>
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<td>Total</td>
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a Mice were given multiple T-AChR/CFA immunizations at monthly intervals. A 5-day IL-12 regimen of 1 μg/day per mouse was also given, accompanying the first two T-AChR immunizations. Myasthenic symptoms were graded using the clinical scale described in Materials and Methods. In addition, a grip strength device with a precision force gauge (Columbus Instruments) was used to measure grams of force of grip. Clinical disease was scored for those mice developing EAMG of stage 2 or higher and with a corresponding drop of >40% in grip strength. The incidence of disease in the transgenic mice was slightly reduced compared with that in the nontransgenic mice. The difference was below statistical significance by the Fisher exact test (p = 0.2). In addition, there was no differential in disease severity as measured by maximum clinical score.
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References


