Breakdown of Tolerance to a Self-Peptide of Acetylcholine Receptor α-Subunit Induces Experimental Myasthenia Gravis in Rats

Fulvio Baggi, Andrea Annoni, Federica Ubiali, Monica Milani, Renato Longhi, Widmer Scaioli, Ferdinando Cornelio, Renato Mantegazza and Carlo Antozzi

J Immunol 2004; 172:2697-2703; doi: 10.4049/jimmunol.172.4.2697
http://www.jimmunol.org/content/172/4/2697

References
This article cites 29 articles, 11 of which you can access for free at: http://www.jimmunol.org/content/172/4/2697.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at: http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Breakdown of Tolerance to a Self-Peptide of Acetylcholine Receptor α-Subunit Induces Experimental Myasthenia Gravis in Rats

Fulvio Baggi,2* Andrea Annoni,* Federica Ubiali,* Monica Milani,3* Renato Longhi,‡ Widmer Scaioli,† Ferdinando Cornelio,* Renato Mantegazza,* and Carlo Antozzi*

Experimental autoimmune myasthenia gravis (EAMG), a model for human myasthenia (MG), is routinely induced in susceptible rat strains by a single immunization with Torpedo acetylcholine receptor (TACHR). TACHR immunization induces anti-AChR Abs that cross-react with self AChR, activate the complement cascade, and promote degradation of the postsynaptic membrane of the neuromuscular junction. In parallel, TACHR-specific T cells are induced, and their specific immunodominant epitope has been mapped to the sequence 97–116 of the AChR α subunit. A proliferative T cell response against the corresponding rat sequence (R97–116) was also found in TACHR-immunized rats. To test whether the rat (self) sequence can be pathogenic, we immunized Lewis rats with R97–116 or T97–116 peptides and evaluated clinical, neurophysiological, and immunological parameters. Clinical signs of the disease were noted only in R97–116-immunized animals and were confirmed by electrophysiological signs of impaired neuromuscular transmission. All animals produced Abs against the immunizing peptide, but anti-rat AChR Abs were observed only in animals immunized with the rat peptide. These findings suggested that EAMG in rats can be induced by a single peptide of the self AChR, that this sequence is recognized by T cells and Abs, and that breakdown of tolerance to a self epitope might be an initiating event in the pathogenesis of rat EAMG and MG.


Copyright © 2004 by The American Association of Immunologists, Inc. 0022-1767/04/$02.00

Received for publication October 7, 2003. Accepted for publication December 8, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was partially supported by the Italian Ministry of Health (Grant RF2002.159). Presented in part by F.B., A.A., F.U., R.L., R.M., F.C., and C.A., at the 10th International Conference on Myasthenia Gravis and Related Disorders, May 29 to June 1, 2002, Key Biscayne, FL.

2 Address correspondence and reprint requests to Dr. Fulvio Baggi, Neurology IV, Neuromuscular Diseases and Autoimmunity, Istituto Nazionale Neurologico Carlo Besta, Milan, Italy; and National Research Center, Institute of Chemistry of Molecular Recognition, Milan, Italy.

3 Current address: Department of Biochemistry, University of Minnesota, Minneapolis, MN 55455.

4 Abbreviations used in this paper: EAMG, experimental autoimmune myasthenia gravis; AChR, acetylcholine receptor; α-BTX, α-bungarotoxin; LNC, lymph node cell; MG, myasthenia gravis; RNS, repetitive nerve stimulation; SI, stimulation index; TACHR, Torpedo acetylcholine receptor. receptor α subunit. Similarly to native AChR, the human AChR α1–210 recombinant protein induced AChR-binding, AChR-modulating, and AChR-blocking autoantibodies in rats when injected once intradermally as an emulsion (7). In this model, T cells recognized the sequence α125–147 of the human AChR α subunit, and this peptide was then used to induce EAMG (8). To our knowledge, no information is available for the myasthenogenic activity of T cell epitopes derived from rats immunized with self AChR. In this paper we describe the induction of EAMG in Lewis rats by a synthetic peptide corresponding to region α97–116 of the rat AChR α subunit. We found that breaking of tolerance to a single T cell epitope of the self autoantigen induces autoreactive T cells and specific Abs to rat AChR, leading to clinical signs of the disease. A similar mechanism might be of relevance to induction of the corresponding disease in humans.

Materials and Methods

Animals

Female Lewis rats, 6–8 wk of age, were purchased from Charles River (Calco, Italy) and housed at the animal facility of the Neurological Institute Carlo Besta (Milan, Italy). Experiments were approved by the ethical committee of the Institute and were performed in accordance with the Principles of Laboratory Animal Care (European Communities Council Directive 86/609/EEC).

Antigens

AChR was purified from Torpedo californica electrophore tissue (Aquatic Research Consultants, San Pedro, CA) by affinity chromatography on Naja naja siamensis toxin coupled to Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden) (9). The TACHR concentration was determined by [125I]-α-bungarotoxin ([125I]-α-BTX; Amersham Italia, Milan, Italy) binding assay, and the specific activity was 3.7–5.5 pmol [125I]-α-BTX binding sites/mg protein. The purified receptor was analyzed on SDS-PAGE. TACHR preparations were dialyzed extensively against 0.01 M Tris-HCl (pH 7.5), 0.001 M EDTA, 0.1M NaCl (Tris-HCl buffer), and 0.1% Triton X-100 and stored at −80°C. TACHR used for T cell proliferations was further dialyzed against Tris-HCl buffer and 0.025% Triton X-100 and filter-sterilized (0.2 μm). The peptides 797–116 (DGDFAIHVHTKLLDDYTGKI) and 97–116 (DGDFAIKVFKTVKLLDDYTGHI) were synthesized according to GenBank published sequences (J00963 for Torpedo, X74832 for rat AChR α
subunit). Peptides were synthesized using F-moc chemistry on an 431A automated peptide synthesizer (PE Applied Biosystems, Foster City, CA). Peptides were purified by reverse phase HPLC: their synthesis was con-

firmed by mass spectroscopy.

**Immunization protocols**

Female Lewis rats (6–8 wk) were immunized in the hind foot pads with 50 μg of 97–116 peptide in CFA (Sigma-Aldrich, Milan, Italy) supplemented with 1 mg of H37Ra/rat (Difco, Detroit, MI) on day 0 and were boosted on day 30 with the same peptide in IFA (Sigma-Aldrich). EAMG was also induced with 50 μg of purified TACr in CFA (plus 1 mg of H37Ra/rat).

**Clinical evaluation**

Each experimental animal was weighed at the beginning of the experiment and twice weekly. The severity of the disease was scored by measuring muscular weakness. Clinical scoring was based on the presence of tremor, hunched posture, muscle strength, and fatigability. Fatigability was as-

sessed after exercise (repetitive paw grips on the cage grid) for 30 s. Dis-

ease severity was expressed as follows: grade 0, normal strength and no abnormalities; grade 1, mildly decreased activity and weak grip or cry, more evident at the end of testing; grade 2, clinical signs present before exercise (tremor, head down, hunched posture, weak grip); grade 3, severe clinical signs present before exercise, no grip, moribund; and grade 4, dead. Results are expressed as the mean of the evaluations recorded for each animal at each time point. EAMG in sick animals was confirmed by edro-

phonium chloride test; repetitive nerve stimulation (RNS) was performed as described previously (10).

**AChR content in rat muscle**

AChR content in muscles was assayed as described previously (11). Briefly, AChR was solubilized from muscle membranes with Tris- HCl buffer and 2% Triton X-100 overnight at 4°C, and the solutions containing solubilized AChR were clarified by centrifugation at 100,000 × g for 30 min. AChR crude extracts (100 μl, duplicates) were incubated with [125I]-BTX for 4 h at room temperature, transferred on DE-81 DEAE disks, and washed with Tris-HCl buffer and 0.5% Triton X-100. Radioactivity was determined by gamma counting. The specific binding was subtracted from each sample by parallel tubes preincubated with cold α-BTX. The results were expressed as picograms of [125I]-BTX binding sites per 100 g of muscle.

**ELISA assay for anti-peptide Ab and isotypes**

A standard ELISA technique was used to detect peptide 97–116-specific Abs. Briefly, 96-well microtiter plates were coated overnight with 5 μg of peptide at 4°C and blocked with 200 μl of PBS, 0.05% Tween 20, and 1% BSA. Twenty-five μl of serially diluted (1/100,000) rabbit anti-rat IgG (HRP-conjugated; Sigma-Aldrich) was added. IgG1, IgG2a, and IgG2b anti-peptide Abs were revealed using mouse anti-rat mAbs (1/250) specific for each subtype (IgG1, IgG2a, and IgG2b; from Sigma-Aldrich, and IgG2c from BD PharMingen, Milan, Italy) for 1 h at room temperature. Plates were washed with PBS/0.05% Tween 20, incubated for 1 h at room temperature with diluted (1/10,000) rabbit anti-2-ME (BDH, Milan, Italy) for 1 h at room temperature. Plates were washed with PBS/0.05% Tween 20, incubated for 1 h at room temperature with diluted (1/10,000) anti-mouse IgG (HRP-conjugated; Sigma-Aldrich), and revealed by o-phenylenediamine substrate. OD was measured at 450 nm using an au-

tomated microplate ELISA reader. Each serum was tested in duplicate and assessed at two different dilutions. Results are expressed as OD at 450 nm per microtiter of serum.

**Anti-AChR Ab assay**

Anti-rat AChR Abs were assayed in individual sera by conventional radioimmunoprecipitation methods (12). Briefly, rat AChR was extracted from denervated rat muscle and labeled with 2 × 10−13 M [125I]-α-BTX. Experimental and control sera were incubated overnight with [125I]-α-BTX rat AChR (0.5 pmol); Ab-AChR complexes were precipitated by adding an excess of rabbit anti-rat IgG (Sigma-Aldrich). The pellets were washed twice with 1 ml of cold PBS/0.5% Triton X-100 and counted in a gamma counter. Serum samples incubated with rat AChR precipitated in an ex-

cess of cold α-BTX (aspecific binding) were subtracted from test samples. The anti-AChR Ab titers were expressed as picograms of [125I]-α-BTX binding sites precipitated per milliliter of serum.

**T cell proliferation assay**

Popolated lymph nodes were aseptically removed and processed into a single-cell suspension. T cells (4 × 10^6) were plated in triplicate in 96-well culture plates (Costar, Cambridge, MA) with the relevant peptide(s) in RPMI 1640 (Sigma-Aldrich) medium with 1% normal rat serum, 1% pyru-

vate, 1% nonessential amino acids, 1% t-glutamine, 1% penicillin-strept-

tomyycin (Euroclone Cellbio, Milan, Italy), and 2 × 10−5 M 2-ME (BDH, Milan, Italy). Con A (Sigma-Aldrich) was used at 2 μg/ml as a positive control. After 72 h of culture, 1 μCi of [3H]thymidine (Amersham Italia) was added, and the plates were harvested after an additional 18 h. Results were expressed as mean counts per minute of triplicate cultures or as stim-

ulation index (SI, the ratio between the mean counts per minute from Ag-

stimulated culture and the mean counts per minute from unstimulated culture).

**Cytokine assay**

Capture ELISAs for the detection of rat IFN-γ, IL-2, IL-6, and IL-10 (R&D Systems Minneapolis, MN) in culture supernatants were performed accord-

ing to the manufacturer’s recommendation. Lymph node T cells (1.5 × 10^5/ml, 24-well plates) were cultured in the presence of 25 μg/ml of the relevant peptide in complete RPMI 1640 medium. Supernatants were har-

vested at the appropriate time for each cytokine and were stored at −80°C pending assay. Each sample was tested in duplicate. Values were expressed as picograms per milliliter of culture medium for each cytokine, calculated from a standard curve.

**Statistical analysis**

The Mann-Whitney U rank test and Student’s t test were used for statistical analysis. The level of significance was p < 0.05.

**Results**

**Characterization of T cell epitopes in Lewis rats**

Different groups mapped T cell epitopes on the α subunit of the AChR in EAMG in Lewis rats, and the identified sequences were closely related to the kind of Ag used to induce EAMG (purified TACr, Torpedo, or human AChR α subunit recombinant pro-

teins). We focused our T cell studies on the sequence correspond-

ing to residues 97–116 of the AChR α subunit, because of two independent reports indicating that sequences 97–116 (6) and [Try^116] [100–116] (4) were overlapping immunodominant T cell epitopes in TACr-induced EAMG in Lewis rats. The region α97–116 differs between Torpedo and rat receptor sequence at positions 104 (His→Lys), 105 (Met→Phe), 108 (Leu→Val), and 115 (Lys→His); 16 of 20 residues are identical (80% homology). Therefore, we wondered whether the sequence R97–116, derived from rat AChR, might be recognized by T cells and represent a self-

t cell epitope in EAMG. Lewis rats were immunized with TACr and sacrificed 1) 10 days after immunization, or 2) when EAMG was evident (7–8 wk after immunization). Lymph node cell (LNC) reactivity against TACr, T97–116, and R97–116 peptides was then evaluated by conventional [3H]thymidine incorporation assay (Fig. 1). As expected, T97–116 peptide was recognized at both time points considered, as it results from processing of the priming Ag (TACr). On the contrary, LNC recognition of R97–116 and R97–116 peptides was then evaluated by conventional [3H]thymidine incorporation assay (Fig. 1). As expected, T97–116 peptide was recognized at both time points considered, as it results from processing of the priming Ag (TACr). On the contrary, LNC recognition of R97–116 and R97–116 peptides was then evaluated by conventional [3H]thymidine incorporation assay (Fig. 1).

**EAMG induction**

EAMG is routinely induced in the Lewis rat by a single immuni-
zation of purified AChR from *Torpedo* electroplax (20 μg) in CFA. The observed cross-reactivity between R97–116 and T97–116 of the AChR subunit.
116 peptides at the T cell level suggested testing these sequences as a potential autoantigen able to induce the experimental disease in Lewis rats. Preliminary experiments were performed to define the optimal dose of Ag; a single immunization was not sufficient to induce clinical manifestations of EAMG, although T cell responses as well as anti-peptide Abs were detected in immunized rats (data not shown). The immunization protocol we thereafter adopted was as follows: one immunization with peptide (50 μg) in CFA in the hind footpads on day 0, followed by a booster injection of peptide (50 μg) in IFA 1 mo later. Three independent experiments were performed, and results are reported in Table I. Evaluation of EAMG was assessed by body weight and clinical score; the disease became evident 8 wk after TACrR immunization.

**FIGURE 1.** Proliferative responses of LNCs derived from TACrR-immunized Lewis rats. LNCs from TACrR-immunized rats were processed into a single-cell suspension and challenged in vitro with R97–116, T97–116, and TACrR for 3 days. Proliferative responses were studied at 10 days (A) and 8 wk (B) after immunization, when EAMG was evident. The dotted line indicates an SI of 3. Recognition of R97–116 peptide became evident 8 wk after TACrR immunization.

AChr content compared with normal control muscles (16.9 ± 10.6 and 40.5 ± 12.1 pmol/100 g, respectively; p = 0.0007); these values were not different from those in rats immunized with TACrR (12.6 ± 3.9 pmol/100 g; p = 0.46). AChr content in muscles from T97–116-immunized rats (35.1 ± 12.6 pmol/100 g) was not statistically different from the amount of receptor in control muscles (p = 0.47).

Presence of Abs against peptides R97–116, T97–116, and rat (self) AChr in EAMG rats

Blood samples were collected at the beginning of each experiment, before the boost (d = 30), and at the end of experiments to evaluate Ab production. Thirty days after immunization with R97–116 and T97–116 peptides (i.e., blood samples taken before boost), Lewis rats developed anti-peptide Ab; anti-R97–116 Ab were 0.189 ± 0.014, and anti-T97–116 Ab were 0.280 ± 0.019, respectively (ΔOD at 450 nm; mean ± SEM; p = 0.0062; Fig. 5, A and B). These values did not change significantly after boosting with the relevant peptide (ΔOD at 450 nm, 0.177 ± 0.016 and 0.297 ± 0.021, respectively; Fig. 5, A and B). The difference between the two groups was still statistically significant (p = 0.01). We also measured the level of Abs to the nonimmunizing peptides. Anti-T97–116 Ab were found in R97–116-immunized animals, and anti-R97–116 Ab were found in T97–116-immunized rats. Their values did not significantly increase after the boost (Fig. 5, A and B). These results suggest that the presence of anti-peptide Abs was not associated with EAMG onset or progression.

We next measured the level of pathogenic anti-rat AChr Ab by a conventional radioimmunoprecipitation assay (Fig. 5C). Thirty days after immunization (before the boost), the mean anti-rat AChr Ab titers were significantly different among experimental groups: 7.7 ± 1.3 pmol/ml (mean ± SEM) in R97–116-immunized rats vs 3.9 ± 1.8 pmol/ml in T97–116-immunized rats (p = 0.023). At this time, overt signs of EAMG were not evident in any animal, although some rats showed slight fatigability. Anti-rat AChr Ab titers, measured at the end of the experiments, were increased in R97–116-immunized rats (22.5 ± 6.3 pmol/ml) compared with pre-boost levels (7.7 ± 1.3 pmol/ml; p = 0.0043) and to levels in the T97–116 group (3.97 ± 0.14; p = 0.014). The low
level of anti-rat AchR Ab in T97–116-immunized rats was not able to impair the neuromuscular transmission, as demonstrated by normal RNS, no clinical signs of EAMG, and normal AchR content. Anti-rat AchR Ab were also measured in the presence of an excess of R97–116 peptide (to saturate anti-peptide Abs present in sera), but we did not observe a statistically significant reduction in anti-rat AchR Ab titers. We did not measure anti-TAChR Ab in peptide-immunized rats because they are not pathogenic; anti-rat and anti-Torpedo AchR Ab titers in TAChR-induced EAMG were 33.5 ± 4.5 and 3630.2 ± 70.5 pmol/ml, respectively.

To determine whether immunization with R97–116 and T97–116 peptides induced different IgG subtypes of anti-peptide Abs by ELISA. OD values were similar for IgG1, IgG2a, IgG2b, and IgG2c in the two groups, with a predominance of the IgG2b type (data not shown); this is probably due to the same protocol of immunization and subsequent boost adopted. Anti-rat AchR IgG subtypes were also determined in R97–116-induced EAMG and compared with TAChR-induced EAMG; again, no significant differences were noted (data not shown).

Recognition of R97–116 and T97–116 peptides by lymph node T cells

Proliferative responses of LNC were assessed when the clinical condition of the sick animals required ending the experiment. Lymph nodes were aseptically removed and cultured in the presence of R97–116 and T97–116 peptides. A strong proliferative response against R97–116 (Fig. 6A) and T97–116 peptide (Fig. 6B), not statistically different from that obtained with Con A (positive control), was observed. LNCs gave proliferative responses when challenged with purified TAChR. Specific recognition of the relevant (immunizing) peptide as well as partial cross-recognition of the nonimmunizing peptide, observed in preliminary experiments reported above (Fig. 2), were again confirmed. Note that recognition of R97–116 in T97–116-immunized rats (Fig. 6B; mean ± SEM, 63,871 ± 18,059 cpm) was significantly different compared with the T97–116 response (122,457 ± 25,639; p = 0.03) and was similar to that obtained in the presence of TAChR (60,462 ± 19,850). In R97–116-immunized rats (Fig. 6A), the specific response to T97–116 (cross-recognition) was lower than that in R97–116-immunized rats, but not statistically different (120,326 ± 27,004 and 166,192 ± 25,679, respectively), whereas the response to T97–116 was significantly different (p = 0.023) compared with that in the presence of TAChR (47,433 ± 14,494). The different degree of recognition of the nonimmunizing peptide suggests that T cells recognized specific residues not shared by the two Ags; this is probably due to a different Ag processing of the two peptides.

Lymph node T cell response to peptide R97–116 is associated with a different pattern of cytokine expression compared with T97–116

To investigate the mechanisms associated with EAMG induction by R97–116 peptide, we evaluated Th1- and Th2-type cytokine

---

### Table I. Incidence of EAMG

<table>
<thead>
<tr>
<th>Rats Immunized with</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
<th>Expt. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EAMG incidence (no. (%))</td>
<td>RNS</td>
<td>EAMG incidence (no. (%))</td>
</tr>
<tr>
<td>Rat 97–116</td>
<td>3/4 (75.0)</td>
<td>2/3 b</td>
<td>4/6 (66.7)</td>
</tr>
<tr>
<td>Torpedo 97–116</td>
<td>0/4</td>
<td>0/4</td>
<td>0/6</td>
</tr>
<tr>
<td>TChR</td>
<td>8/9 (88.9)</td>
<td>8/9</td>
<td>ND</td>
</tr>
</tbody>
</table>

**Mean clinical score**

- Expt. 1: 2.5 ± 0.35
- Expt. 2: 0
- Expt. 3: 3 ± 0.26

---

a One rat died of EAMG before neurophysiological evaluation.
b ND, Not done.
c ND was not performed in experiment 3.

---

**FIGURE 3.** EAMG clinical score (A) and variations in body weight (B) in TACHR-, R97–116-, and T97–116-immunized rats. Clinical manifestations of EAMG were evident only in R97–116-immunized rats (■); T97–116-immunized rats (△) did not develop EAMG, and their growth curve is similar to that of normal rats (not plotted). Representative data are from experiment 1; the arrows indicate the booster injection with R97–116 or T97–116 peptide. Note the different time course of onset and severity of EAMG between TACHR-immunized (○) and R97–116-immunized rats.
release in culture supernatants of lymph node T cells stimulated in vitro with the same peptide used for immunization. We considered IFN-γ and IL-2 as markers for the Th1 subpopulation, and IL-6 and IL-10 as markers for the Th2 subpopulation. Data are reported in Table II. We did not observe any significant modification of Th1-type cytokine levels in supernatant from R97–116-stimulated LNCs derived from R97–116-immunized rats compared with T97–116 stimulated LNCs taken from T97–116-immunized rats. On the contrary, a significantly lower production of IL-6 and IL-10 in the R97–116 group was measured (91% reduction for IL-6, p = 0.0017; 76% reduction for IL-10, p = 0.0013). These data suggest that EAMG induction by R97–116 peptide might be associated with an unbalanced production of cytokines toward the Th1 compartment (i.e., the observed reduction in Th2 compartment implies a functional predominance of the Th1 response in R97–116-immunized rats).

Discussion

EAMG is routinely induced using xenogenic AChR purified from Torpedo electric tissue (13). TACHR-induced EAMG in susceptible strains (such as Lewis rat) is characterized by high incidence, rapid manifestation of the disease, and quick progression. Clinical signs of the disease are due to anti-TACHR Abs cross-reacting with the self (rat) AChR on the neuromuscular junction. The native receptor contains multiple antigenic determinants, both conformational (B cell epitopes) and linear (T cell epitopes), that are highly effective in activating the host immune system. As occurs in the human disease, Ab production in EAMG is thought to be under the control of AChR-specific T cells. The identification of immunodominant AChR sequences relevant for the T or B cell compartments that are effective in inducing EAMG might be of help in understanding the key immunological processes leading to the disease and hence improve our knowledge of the pathogenesis of MG.

In some experimental models, myasthenogenic sequences were identified among B cell epitopes, and these regions were located within the main immunogenic region (a67–76) (14) or near the α-BTX binding site-acetylcholine binding site: α125–147 (15) and α183–200 (16). Anti-peptide Abs, able to bind to conformational extracellular domains of the AChR, can directly attack the receptor or interfere with the acetylcholine binding site. Indeed, rats immunized with α125–147 derived from Torpedo sequence, either with or without the disulfide link Cys128–Cys142, produced anti-peptide Abs that bound to the native receptor (17, 18). EAMG in rats has been induced by α183–200 peptide derived from Torpedo, but not from human AChR α subunit sequence (16); these sequences differ in three residues at positions 187, 189, and 191, and the researchers suggested that AChR-blocking Abs were induced. Artificial peptides were synthesized by coupling peptides α67–76 or α190–195 (B cell epitopes) and peptide α107–116 (T cell epitope) with spacing residues (Asn-Pro-Gly-Gly). Lewis rats received multiple injections on days 0, 14, and 28 and were killed on...
T cells from TAChR-immunized Lewis rats recognized the immunodominant sequences [Tyr100]α100–116 or [Gly89,Tyr90]α73–90 (4) and region α97–112 (5, 6) of Torpedo AChR α subunit. T cells primed with these peptides showed significant proliferation when challenged in vitro with either the homologous peptide or intact AChR. When peptides [Tyr100]α100–116 and [Gly89,Tyr90]α73–90 were used as immunogen, none of the rats showed clinical signs of EAMG (4).

On the contrary, when Lewis rats were immunized with the recombinant extracellular portion of the human (mammalian) AChR α subunit (α1–210), T lymphocytes preferentially proliferated in response to peptide α125–147 of the human receptor (7). This epitope was further refined and shortened to α129–145 (8), a sequence identical with rat AChR. In this model, Bordetella pertussis was used as supplementary adjuvant to CFA, and rats were repeatedly immunized (two to five times) with this peptide. Clinical signs of EAMG were evident in only three of 23 rats immunized with α125–147, but in none of the rats immunized with α129–145 (8). This immunodominant peptide was also studied by Matsuo et al. (21), but they did not observe clinical signs of the disease in their animals that received only a single injection of peptide in CFA supplemented with B. pertussis.

Our data differ from the studies reported above. A high incidence of the disease was observed when rats were immunized with the self peptide (>70% of rats developed EAMG in the R97–116-immunized groups). EAMG was clearly evident and confirmed by RNS, loss of body weight, and positive EAMG clinical score. The mean EAMG score observed at the end of the experiments was comparable to that usually observed after immunization with TACHR. The model was reproducible, as clinical signs of EAMG were observed in three independent experiments. Moreover, i.p. administration of edrophonium chloride was able to reverse EAMG symptoms in all sick animals. Elevated anti-rat AChR Abs and reduced AChR content in muscles were found only in R97–116-immunized rats. Anti-rat AChR Abs present at low level in T97–116-immunized rats were not able to interfere with the neuromuscular transmission, as demonstrated by normal RNS, no clinical signs of EAMG, and normal muscle AChR content in these animals. It should be considered that immunization of animals started at 8 wk of age compared with 10–14 wk in a previous study (8). The low incidence of clinical EAMG in the model induced by α129–145 can be partially explained by the lower susceptibility to disease induction of older rats (22).

Anti-AChR Abs are characterized by high affinity and specificity for the intact conformation of the Ag; Abs that are directed against extracellular AChR domains are directly pathogenic, whereas those that are specific for unaccessible domains (intracellular or intrachain) are probably not directly involved in degradation of the neuromuscular junction. We believe that anti-peptide Abs induced in our model belong to the second type, because region 97–116 encompasses the β5 and β5’ domains of the AChR α subunit, that seem to be (at least partially) hidden and in close contact with the ε subunit (23).

In mouse models of EAMG, pathogenic anti-AChR Abs belong to the IgG2a subclass and are associated with Th1-type responses. Although the anti-TAChR Ab response is IgG2b dominant in Lewis rats and IgG1 dominant in Brown-Norway rats, both strains are susceptible to EAMG (24) as all IgG subclasses are capable of binding complement and promote degradation of the neuromuscular junction. We wondered whether the IgG subclass composition of anti-peptide Abs might be taken into account for EAMG induction in R97–116-immunized rats, but we did not observe any difference.

Yeh et al. (25) studied the immune reactivity to peptide α100–116 from TACHR α subunit, and their data proved that T cells reactive with this peptide can provide help for the anti-AChR Ab

---

**Table II. In vitro Th1 and Th2 cytokines production**

<table>
<thead>
<tr>
<th>Condition</th>
<th>IFN-γ (pg/ml)</th>
<th>IL-2 (pg/ml)</th>
<th>IL-6 (pg/ml)</th>
<th>IL-10 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNCs from R97-116 immunized rats</td>
<td>2949.1 ± 278.3</td>
<td>228.5 ± 45.4</td>
<td>3.1 ± 2.3</td>
<td>36.4 ± 9.7</td>
</tr>
<tr>
<td>LNCs from T97-116 immunized rats</td>
<td>3466.3 ± 133.9</td>
<td>147.7 ± 8.4</td>
<td>34.2 ± 6.5</td>
<td>150.8 ± 21.8</td>
</tr>
</tbody>
</table>

* p = NS.
* p = 0.0017.
* p = 0.0013.
response; moreover, they suggested that the level of T cell help (to Ab production) can be separated from T cell proliferation in response to α100–116 variants with proline or threonine at position 106. It must be noted that the sequence they studied is derived from TaChR, but with Tyr at position 100, whereas published sequences are characterized by Phe\(^{100}\), as were the rat and Torpedo \(97–116\) peptides we used. We have not yet performed specific experiments to evaluate whether Tyr\(^{100}\) or Phe\(^{100}\) substitution might be relevant for immune sensitization to AChR. Moreover, amino acid residues that differ between rat and Torpedo α97–116 sequences (His→Lys, position 104; Met→Phe, position 105; Leu→Val, position 108; and Lys→Hys, position 115) need to be studied at the single amino acid level, looking for those residues that might be crucial in terms of peptide presentation or recognition by T cells and subsequently for help to B cells. Indeed, peptide α97–116 contains the proposed binding residues for the rat MHC class II molecule RT1.B\(^{1}\) (26). We cannot exclude that our rat and torpedo peptides (that are four residues longer than the described peptide 101–116) could bind to rat MHC with different anchor residues or frames. Alternatively, processing of our 20-mer peptides might generate shortened sequences with different affinities to class II molecules. Hence, AChR-reactive T cell clones with different patterns of cytokine production or help in Ab production might be generated.

It has been demonstrated that muscle cells are able to present epitopes derived from the AChR to specific CD4\(^{+}\) T cells. These T cells could, in turn, initiate or maintain the anti-AChR response that is responsible for AChR loss in MG. More interestingly, T cell activation results in IFN-γ secretion and killing of the myoblast (27, 28). Indeed, a reduced Th2 response with a relative unbalance toward the Th1 compartment was found in cultured LNCs from our R97–116-immunized rats. Th2 reduction was not accompanied by any modification in the pattern of IgG subclass composition of anti-peptide Abs. We suggest that this unbalanced Th1/Th2 response affected only the T cell arm of the autoimmune response. We propose that 1) both Abs and T cells specific for R97–116 are induced in our model, but 2) R97–116-specific Abs do not exert any myasthenogenic activity because this sequence is hidden in AChR structure, and 3) R97–116-specific T cells might recognize the self 97–116 peptide (a naturally processed immunodominant T cell epitope) presented by muscle cells. Activated T cells might exert cytotoxic activity toward the APCs, resulting in the release of native AChR and hence in the breakdown of tolerance to AChR and EAMG. This hypothesis has also been proposed to explain the role of T cells in the initiation of autoantibody response in MG, at least in patients with thymic abnormalities (29).

Our data indicate that EAMG can be induced by a single peptide of the self AChR, inducing an Ag-specific immune response. Clinical and neuropathological signs of EAMG as well as anti-rat AChR Abs were found in R97–116-immunized rats only. Sequence α97–116 of the rat AChR α subunit plays a role in the pathogenesis of the disease in Lewis rats, and the primary event might be the breakdown of self tolerance at the T cell level. Antigenic segments of the self AChR might constitute a target for Ag-specific immunotherapeutic strategies of MG.

References

11. Lindstrom, J. M., B. Einarson, V. A. Lennon, and N. E. Seybold. 1976. Pathological mechanisms in experimental autoimmune myasthenia gravis. I. Immunogenicity of syngeneic muscle acetylcholine receptor and quantitative extraction rate of receptor and antibody receptors. Clin. Exp. Immunol. 21:116-immunized rats. Th2 reduction was not accompanied by any modification in the pattern of IgG subclass composition of anti-peptide Abs. We suggest that this unbalanced Th1/Th2 response affected only the T cell arm of the autoimmune response. We propose that 1) both Abs and T cells specific for R97–116 are induced in our model, but 2) R97–116-specific Abs do not exert any myasthenogenic activity because this sequence is hidden in AChR structure, and 3) R97–116-specific T cells might recognize the self 97–116 peptide (a naturally processed immunodominant T cell epitope) presented by muscle cells. Activated T cells might exert cytotoxic activity toward the APCs, resulting in the release of native AChR and hence in the breakdown of tolerance to AChR and EAMG. This hypothesis has also been proposed to explain the role of T cells in the initiation of autoantibody response in MG, at least in patients with thymic abnormalities (29).

Our data indicate that EAMG can be induced by a single peptide of the self AChR, inducing an Ag-specific immune response. Clinical and neuropathological signs of EAMG as well as anti-rat AChR Abs were found in R97–116-immunized rats only. Sequence α97–116 of the rat AChR α subunit plays a role in the pathogenesis of the disease in Lewis rats, and the primary event might be the breakdown of self tolerance at the T cell level. Antigenic segments of the self AChR might constitute a target for Ag-specific immunotherapeutic strategies of MG.

References