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R Hohlfeld, ... , S L Walgrave, B M Conti-Tronconi

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Research Article

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Amphipathic Segment of the Nicotinic Receptor Alpha Subunit Contains Epitopes Recognized by T Lymphocytes in Myasthenia Gravis

Reinhard Hohlfeld,* Klaus V. Toyka,* Lucinda L. Miner,† Susan L. Walgrave,† and Bianca M. Conti-Tronconi†

*Department of Neurology, University of Duesseldorf, Duesseldorf, Federal Republic of Germany; and †Department of Biochemistry, University of Minnesota, St. Paul, Minnesota 55108

Abstract

Autoimmune helper T lymphocytes were selected from the blood of two myasthenic patients of different HLA-DR type, using acetylcholine receptor (AChR) from *Torpedo californica*. These polyclonal T cell lines were tested for reactivity with three synthetic peptides corresponding to the NH₂-terminal region of the human AChR alpha subunit. This segment is a good candidate for T cell epitopes since it has a propensity to form an amphipathic alpha helix. The peptides elicited 10–30% of the response induced by native *Torpedo* AChR. Different peptides were recognized by the autoreactive T cells of the two patients. These results suggest that the NH₂-terminal region of the AChR alpha chain contains T cell-stimulating epitopes, and that the T cell autoimmune response in myasthenia gravis, like the B cell response, is heterogeneous.

Introduction

Myasthenia gravis (MG)¹ is characterized by failure of the neuromuscular transmission. This is mainly caused by a loss of functional acetylcholine receptors (AChRs), induced by binding of anti-AChR antibodies (reviewed in reference 1). The AChR is a complex transmembrane protein formed by four homologous subunits in a stoichiometry $\alpha_2\beta\gamma\delta$ (2, 3). The amino acid sequence of AChR subunits from different tissues and animals is known. Their structure is highly conserved, and subunits of corresponding molecular weight from different species are very similar. The alpha subunit dominates the anti-AChR antibody response in MG. A substantial fraction of antibodies bind to a discrete area of the alpha subunit, called the "main immunogenic region" (4). However, the B cell response is clearly polyclonal, and even the anti-main immunogenic region antibodies do not have a predominant idio type (5). These observations have dampened the initial optimism about the therapeutic potential of anti-idiotypic antibodies. Since the anti-AChR antibody synthesis is regulated by AChR-specific T helper cells (6–8), these T cells are another possible target for immunotherapeutic intervention (9). It is therefore important to understand how the autoimmune B

cells and the corresponding helper T cells recognize the same complex antigen, how their recognition is influenced by the class II histocompatibility molecules expressed by the patient, and how these two cell compartments interact with each other.

We have recently found that the majority of the epitopes recognized by AChR-specific T helper cells are present on the denatured alpha subunit (8). The present study is a first step in an effort to identify the segments of the alpha subunit that form epitopes recognized by the helper T cells, and to elucidate how diverse the T cell response is in different patients.

Methods

Patients. AChR-reactive T cell lines were isolated from two patients who had generalized MG according to clinical, pharmacological, and electrophysiological criteria. The serum concentration of anti-AChR antibodies (measured with a modified radioprecipitation assay (10) and expressed as nanomoles alpha-bungarotoxin-binding sites per liter of serum) was 2.0 in patient 1 (40 yr; male), and 1,690.0 in patient 2 (20 yr; female). The patients had not been taking immunosuppressive medications for at least 3 mo before the time when the T cell cultures were started (see below). HLA types, determined with standard microlymphotoxicity techniques, were *Aw24,A28/Bw35,Bw51/Cw3,-/DR1,DR4* (patient 1) and *A1,A31/B8,-/Cw7,-/DR3,DRw8* (patient 2).

Isolation of AChR-reactive T cell lines. AChR-reactive T cell lines were obtained from the peripheral blood as described previously (6–8). Briefly, PBMC were initially cultured in bulk cultures in the presence of 5 μ g/ml *Torpedo* AChR. After 4–6 d the reactive lymphoblasts were separated from the small, resting lymphocytes by centrifugation on Percoll density gradients (Pharmacia Fine Chemicals, Div. of Pharmacia, Uppsala, Sweden) and were propagated further by repeated stimulation cycles with IL-2 (Lymphocult-T LF, Biotest, Frankfurt, FRG) followed by 5 μ g/ml *Torpedo* AChR plus mitomycin C-treated autologous or HLA-matched PBMC as antigen-presenting cells. These polyclonal CD3,CD4-positive helper T cell populations were analyzed for antigen fine specificity as soon as a satisfactory enrichment in AChR reactivity was achieved, i.e., after ~ 4 wk of culture (see Results). The T cells from patient 2 were isolated early in 1987 and were used freshly for fine specificity analysis (see below). The T cells from patient 1 were isolated in 1983, before the patient had a clinical relapse necessitating the institution of immunosuppressive therapy. After continuous propagation in culture until 1984, these cells were frozen in RPMI 1640 (Gibco Europe, Gibco Div., Karlsruhe, FRG), containing 10% DMSO and 20% FCS (Gibco Europe), and were maintained in liquid nitrogen until use in 1987.

Peptide synthesis and characterization. Three peptides, each between 14 and 16 residues long and having 4–8 overlapping residues, were synthesized by manual parallel synthesis (11). The minimum amount obtained for each peptide was 5 mg. These peptides correspond to the amino-terminal 34 residues of the human AChR alpha subunit sequence (Fig. 1). Computer analysis of the total alpha subunit sequence for propensity to form alpha amphipathic helices (12, 13) had indicated that this segment has the highest degree of periodic amphipathicity both in the human and *Torpedo* alpha subunit (unpublished results; obtained and kindly made available to us by Drs. James Cornette, Hannah Margalit, Charles DeLisi, and Jay A. Berzofsky). The

Dr. Hohlfeld's present address is Neuromuscular Research Laboratory, Mayo Clinic, Rochester, MN 55905.

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1. *Abbreviations used in this paper:* AChR, acetylcholine receptor; MG, myasthenia gravis.

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Figure 1. Amino acid sequence of the human AChR alpha subunit. The three overlapping NH₂-terminal peptides used for T cell stimulation are designated P1–P3. M1–M4 denote four of the presumably five transmembrane regions (see reference 2 for review). The carboxy-terminal intracellular sequence is at the bottom.

purity of the synthetic peptides ranged between 65 and 85%, as assessed by reverse phase HPLC using a C18 column (Ultrasphere ODS), and a gradient of acetonitrile in 0.1% trifluoroacetic acid in water. The sequence of the peptides was examined by amino acid analysis, using phenylthiocarbamyl derivatives of the amino acids released by acid hydrolysis (14).

Proliferation assay (unselected PBMC). PBMC were isolated by centrifugation over Ficoll-Hypaque gradients (Pharmacia Fine Chemicals), washed in Hepes-buffered RPMI 1640 (Gibco Europe), and cultured in triplicate in round bottom microtiter plates (Nunc, Roskilde, Denmark) at a concentration of 2×10^5 cells in 0.2 ml of culture medium composed of RPMI 1640 (Gibco Europe) supplemented with 10% heat-inactivated (56°C, 30 min) human AB serum (Gibco Europe), 2 mM L-glutamine, 100 U/ml penicillin, and 50 µg/ml streptomycin. The following antigens (or lectins) were used: purified protein derivative (tuberculin) (Behring, Marburg, FRG; 1 µg/ml), tetanus toxoid (Behring; 1 µg/ml), solubilized Torpedo AChR (8) (5 µg/ml), synthetic peptides of the human AChR alpha subunit (see above; 1 µg/ml), and PHA (Wellcome Reagent Ltd., Beckenham, England; 1 µg/ml). After 72 h the cultures were pulsed with [³H]thymidine (1 µCi/well; sp act 5 Ci/mmol; Amersham International, Amersham, England). 16 h later the cells were harvested with a multiple cell harvester (Titertek, Elfab Oy, Finland), and thymidine incorporation was measured in a liquid scintillation counter. The results were expressed as cpm [³H]thymidine uptake (mean of triplicate cultures ± SD).

Proliferation assay (AChR-reactive, selected T cells). AChR-reactive T cells were cultured and labeled as described above for unselected

PBMC, except that the concentration of the responding T cells was only 1×10^4 cells/0.2 ml/microwell, and that 2×10^5 HLA-matched (patient 2) or autologous (patient 1) mitomycin C-treated PBMC were added into each well as antigen-presenting cells.

Results

Antigen reactivity of unselected PBMC. Table I shows the reactivity of unselected peripheral blood cells in the absence of antigen or with different antigens that were used when the primary cultures for the selection of AChR-specific T cells were started in 1983 (patient 1) and in 1987 (patient 2). Reactivity with tuberculin was detectable in patient 1, and with tetanus toxoid in patient 2. However, there was no significant reactivity with solubilized Torpedo AChR at the optimal stimulating concentration established in previous experiments (5 µg/ml; ~ 20–25 nM) (4, 8). Reactivity with the synthetic peptides P1–P3 (cf. Fig. 1), which was tested in patient 2, was also within or slightly below the background range.

Antigen reactivity of AChR-specific T cells. Fig. 2 shows the AChR response of the CD3,CD4-positive T cell lines that were derived from these PBMC by in vitro selection with solubilized Torpedo AChR (see Methods section). In this experiment the number of responding cells per culture well was only 1×10^4 compared with 2×10^5 in the case of unselected PBMC. In patient 1 (HLA-DR1,4), the AChR-reactive selected T cells were stimulated significantly in the presence of peptides P2 and P3 (Fig. 2, top). After subtracting the background counts, peptides P2 and P3 evoked roughly 30% of the response obtained with solubilized native Torpedo AChR. The selected helper T cells from patient 2 (HLA-DR3,8) did not at all recog-

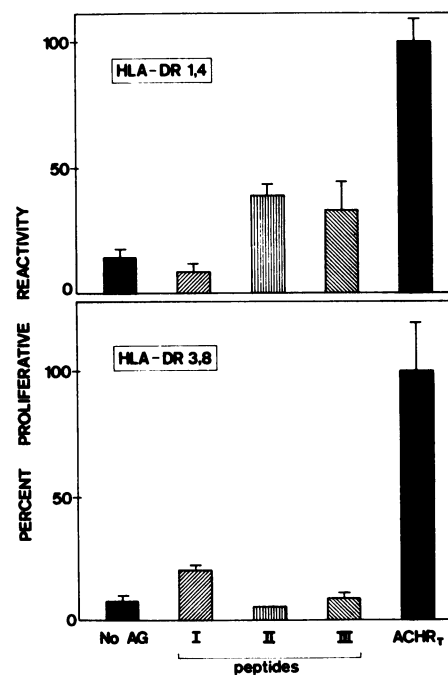


Figure 2. Peptide reactivity of selected, AChR-specific T cells. Top: patient 1 (HLA-DR1,4); bottom: patient 2 (HLA-DR3,8). Results are expressed as percent of the proliferative response ([³H]thymidine uptake; mean of triplicate cultures ± SD) in the presence of 5 µg/ml solubilized Torpedo AChR (AChR₇). No AG (antigen): background proliferation in the absence of antigen. Absolute counts (100%) were 5,260 ± 670 (patient 1) and 12,980 ± 1,760 (patient 2).

Table I. Antigen Reactivity of Unselected PBMC*

Antigen	Patient 1	Patient 2
No antigen	3,033±380	1,172±408
PPD	6,085±1,217	1,168±201
Tetanus toxoid	NT	6,231±690
PHA	67,623±3,245	32,928±3,580
Native Torpedo AChR	3,810±953	1,309±445
Peptide 1	NT	1,219±304
Peptide 2	NT	1,125±467
Peptide 3	NT	747±529

NT, not tested.

* cpm [³H]thymidine uptake (mean of triplicate cultures ± SD).

nize peptides P2 and P3 (Fig. 2, bottom). A small but significant response was detected against peptide P1 (~ 10% of the response induced with native solubilized Torpedo AChR). The optimal stimulating concentration was ~ 1 µg/ml (0.5 µM) for all three peptides. As can be seen from Table I and by comparing the upper and lower panels in Fig. 2, the peptides had no significant unspecific stimulatory or inhibitory effects at this concentration.

Discussion

Three peptides of ~ 2,000 mol wt induced between 10 and 30% of the proliferative response evoked in the same pluriclinal T cells with intact AChR, which has a molecular weight of ~ 270,000 (2, 3). This suggests that the T helper cells recognized a limited number of AChR epitopes. These findings also indicate that other T cell-stimulating epitopes must exist on the AChR molecule. Since most of the determinants recognized by autoimmune T helper cells are located on the alpha subunit (8), the additional, as yet undefined, epitopes are positioned between the end of peptide P3 and the carboxy-terminal end of the alpha subunit (Fig. 1). This conclusion is supported by recent findings of other groups who have evidence for at least two additional distinct T cell-stimulating epitopes that are carboxy-terminal to sequence 1-34 (Lawrence Steinman, personal communication, and reference 15, 16). It is likely that several different T cell clones, each recognizing different sites on the alpha subunit, participate in the autoimmune reaction against AChR. This would argue against the possibility that MG is initiated by one single "forbidden clone" (17) of autosenitized T helper cells.

The autoreactive T cells studied here reacted to peptides which represent one of the sections of the alpha subunit pre-

dicted to be particularly immunogenic for T cells (see Methods). The sequences of these peptides display a high degree of periodicity in hydrophobic residues, and have a propensity to form an amphipathic alpha helix. Such structure could be favored and stabilized by the amphipathic environment at the surface of the antigen-presenting cell. This does not require that the native secondary structure of the intact protein is also alpha-helical (12, 13). Most of the known helper T cell epitopes show a propensity to form amphipathic helices (reviewed in references 18, 19). Our present results indicate that this also applies to a set of epitopes on the NH₂-terminal alpha subunit of the AChR molecule.

Since our T cell lines were selected in vitro with Torpedo AChR, they do not necessarily represent the in vivo repertoire. However, there is evidence that the extent of cross-reactivity between Torpedo and human AChR is high at the level of unselected T cells (reference 8 and unpublished observations). This would indicate that Torpedo AChR is a reasonable substitute for human AChR in the selection of autoreactive human T cells. Table II compares the sequence of the amino-terminal 34 amino acids of the human and Torpedo AChR alpha chain, i.e., the segment that is represented by the three peptides used to stimulate the T cell lines. The amino acid substitutions, some of which are highly conservative, do not alter the amphipathic character of this segment. It has the highest amphipathic score (13) of all amphipathic sequences both in the human and Torpedo AChR alpha chain (the calculated amphipathic score is 55.4 for the human and 56.1 for the Torpedo NH₂-terminal segment).

It is tempting to speculate that the T cell epitopes are represented by the sequences that are shared between the human and Torpedo AChR. However, one must take into consideration that it is unknown how individual amino acid substitutions affect the integrity of either the T cell epitope or the "agretope" (the site interacting with the restricting histocompatibility molecule (20)). The epitope and the agretope may not be laterally separable and may be composed of interspersed amino acid residues (18, 19, 21). Moreover, the agretope and the T cell epitope may differ in their sensitivity to amino acid substitutions (22). To identify these sites it will be necessary to develop clones of AChR-specific T cells and to systematically test their reactivity against panels of substituted peptides.

The extent of clonal diversity of T cell autoimmune reactions is a key issue with regard to possible clinical applications for immunotherapy. Our data indicate that (a) the anti-AChR T cell autoimmune response, although limited in its clonal diversity, is nevertheless heterogeneous in individual patients (intra-individual heterogeneity), and that (b) it varies between different patients (inter-individual variability). With regard to intra-individual heterogeneity of the T cell autoimmune re-

Table II. Comparison of the Amino-Terminal Sequence of the Human and Torpedo californica AChR Alpha Subunit

	1	5	10	15	20	25	30																											
Human:	<u>S</u>	<u>E</u>	<u>H</u>	<u>E</u>	<u>T</u>	<u>R</u>	<u>L</u>	<u>V</u>	<u>A</u>	<u>K</u>	<u>L</u>	<u>F</u>	<u>K</u>	<u>D</u>	<u>Y</u>	<u>S</u>	<u>S</u>	<u>V</u>	<u>V</u>	<u>R</u>	<u>P</u>	<u>V</u>	<u>E</u>	<u>D</u>	<u>H</u>	<u>R</u>	<u>Q</u>	<u>V</u>	<u>V</u>	<u>E</u>	<u>V</u>	<u>T</u>	<u>V</u>	<u>G</u>
					*	*																												
Torpedo:	<u>S</u>	<u>E</u>	<u>H</u>	<u>E</u>	<u>T</u>	<u>R</u>	<u>L</u>	<u>V</u>	<u>A</u>	<u>N</u>	<u>L</u>	<u>L</u>	<u>E</u>	<u>N</u>	<u>Y</u>	<u>N</u>	<u>K</u>	<u>V</u>	<u>I</u>	<u>R</u>	<u>P</u>	<u>V</u>	<u>E</u>	<u>H</u>	<u>H</u>	<u>T</u>	<u>H</u>	<u>F</u>	<u>V</u>	<u>D</u>	<u>I</u>	<u>T</u>	<u>V</u>	<u>G</u>

The sequences (taken from reference 24) are shown by the one-letter amino acid notation. Shared residues are underlined. Conservative substitutions are marked with an asterisk.

sponse, recent results in experimental autoallergic encephalomyelitis are relevant. Zamvil et al. (23) showed that only a very limited repertoire of autoaggressive T cells induce experimental autoallergic encephalomyelitis, and that only a subset of the T cells that recognize the autoantigen, myelin basic protein, is encephalitogenic. By analogy, it is possible that not all of the helper T cells that recognize AChR in a given patient are relevant for the disease because they do not help in the production of myasthenogenic anti-AChR autoantibodies. Therefore, even in a heterogeneous population of autoimmune T cells, it may be possible to define a small number of T cell clones against which specific therapy could be targeted (9).

Interindividual variability of the autoimmune response could be partially explained by the fact that T helper cells recognize antigen in association with class II histocompatibility antigens (MHC restriction; reviewed in reference 20). It is well established that these "restricting" histocompatibility molecules influence T cell responsiveness, and that the immunodominance of particular antigen epitopes depends strongly on the MHC antigens used as restriction elements (reviewed in references 18, 20). Further analyses in this system may help to better define the suspected relationship between HLA class II molecules expressed by individual patients and the antigenic fine specificity of their autoimmune T cells.

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