A Juxta-Membrane Epitope on the Human Acetylcholine Receptor Recognized by T Cells in Myasthenia Gravis

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Abstract

T cell proliferative responses to synthetic peptides taken from the human nicotinic acetylcholine receptor (AChR) α-chain sequence, or to whole AChR purified from electric fish (*Torpedo marmorata*), have been studied, using blood, thymus, and lymph node cells, from 34 patients with myasthenia gravis (MG) and 17 controls mostly with other neurological diseases. Peptides were selected because they contained amino acid motifs that recur in most defined T cell epitopes. Peptide 257–269 (from the extracellular loop of the AChR α-chain between the second and third trans-membrane domains) stimulated cells from six patients and no controls. Peptides from region 125–143 (from the main extracellular 1–210 stretch), which is thought to be an important T cell epitope in rats, provoked responses in 26% of patients and 41% of controls. Two patients responded both to these peptides and to peptide 257–269, thereby implying some heterogeneity of their reacting T cells.

Whereas the initial blood T cell samples sometimes responded both to Torpedo AChR and to the 125–143 peptides, T cell lines selected with either antigen subsequently showed no response to the other. This observation suggests that it may be essential to use human AChR sequences for studying truly autoreactive T cells in MG. Finally, no strong association was found between any of the responses to peptides and the HLA types of the responding individuals.

Introduction

Myasthenia gravis (MG)[1] is an autoimmune disease in which antibodies are produced that bind to acetylcholine receptors (AChR) in the postsynaptic membrane at the neuromuscular junction (1). This leads to loss of receptors and muscle weakness. Production of anti-AChR antibodies by B cells is T cell-dependent in experimental animals (2), and this is also likely to be the case in MG patients. Recently, AChR-specific T cell lines have been isolated from peripheral blood and thymus tissue of MG patients (3, 4).

The AChRs found at the neuromuscular junction are pentameric structures comprising $\alpha_2, \beta, \gamma, \delta$ subunits (1). Approximately 60% of anti-AChR antibodies found in MG sera are directed at conformational determinants on the two α-subunits known as the main immunogenic region (MIR) (5) that probably consists of several nonlinear sites on the extracellular surface. Recent evidence suggests that the majority of AChR-specific T cells also recognize epitopes on the α-subunit (6). Inasmuch as T cells usually respond to sequential determinants rather than the native conformation of the stimulating antigen (7), short peptides may be sufficient provided that they contain the components necessary to bind to the T cell receptor and interact with self MHC molecules. It has been proposed by Rothbard and Taylor (8) that the majority of T cell epitopes contain within their structure a common motif of four or five amino acids in the following sequence: (1) charged or glycine, (2) hydrophobic, (3) hydrophobic, (4) charged, polar or hydrophobic. If the fourth residue is hydrophobic, the fifth must be charged or polar. The exact location of the T cell epitopes on the human AChR that initiate the pathogenic autoimmune reaction in MG is presently unknown. Their identification might provide clues about possible provoking agents in MG as well as about the repertoire of the T cell receptors that recognize them. The present study attempts to identify these sites on the α-subunit using synthetic human peptide sequences containing the common motifs described above.

Methods

Lymphocytes. Peripheral blood lymphocytes (PBL) were obtained from 34 MG patients (33 white, 1 black). 25 had disease onset before the age of 40 yr ("young onset"), 5 after 40 ("old onset"); a further 4 patients had a thymoma. Only two patients were receiving immunosuppressive drug treatment at the time of study. Controls comprised three cases of multiple sclerosis, one patient with cerebellar syndrome in association with ovarian cancer (a condition in which autoantibodies to cerebellar Purkinje cells are present in serum), five with paraproteinemic peripheral neuropathy, four with the Lambert-Eaton myasthenic syndrome (LEMS), and four healthy laboratory workers.

Lymphocytes were separated from heparinized blood by centrifugation on Ficoll-Hypaque, washed in HBSS (Gibco, Grand Island, NY) three times, and then resuspended in RPMI 1640 (Gibco) containing antibiotics and 10% human A^+ serum.

Thymic lymphocytes were also obtained by mechanical disruption (9) from seven of the MG patients undergoing therapeutic thymectomy. In most cases, low-density fractions from discontinuous Ficoll-Hypaque gradients were used (10). Mediastinal lymph nodes from three of these patients were teased apart and the cells were then washed and diluted in RPMI 1640 plus 10% human A^+ serum.

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1. *Abbreviations used in this paper:* AChR, acetylcholine receptor; LEMS, Lambert-Eaton myasthenic syndrome; MG, myasthenia gravis; MIR, main immunogenic region; SI, stimulation index; S–S, disulphide bond.

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Table I. Amino Acid Sequences of 12 Peptides from the AChR α-Subunit

Position	Species	Amino acids
1–15	Human	SEHETRLVAKLFKDY
84–98	Human	KIWRPDLVLYNNADG
99–116	Human	DFAIVKFTKVLLQYTGHI
125–143	Human	KSYSEIIVTHFPFDEQNCS
129–143	Human	EIIVTHFPFDEQNCS
125–143 (disulphide linked)	Human	KSYCEIIVTHFPFDEQNCS
185–196	*Torpedo*	KHWVYYTCCPDT
195–212	Human	DTPYLDITYHFVMQRLPL
257–269	Human	LLVIVELIPSTSS
310–327	Human	NWVRKVFIDTIPNIMFFS
373–389	Human	KSAIEGIKYIAETMKSD
422–437	Human	TLAVFAGRLIELNQQG

Underlined sequences refer to the T cell motifs of Rothbard and Taylor (8).

Peptides. 11 different synthetic peptides representing sequences of the human α-subunit of the AChR, and one from the *Torpedo marmorata* sequence (representing the major site of α-bungarotoxin binding) were prepared on a peptide synthesizer (model 430A, Applied Biosystems Inc., Foster City, Ca.) using commercially available reagents as described (11). The purity of each peptide was analyzed by amino acid analysis and analytical high performance liquid chromatography (HPLC) on a C-18 reverse-phase column and ranged from 72% to 96% purity. The sequences of these peptides are shown in Table I. Peptides were dissolved in deionized water, sometimes requiring the addition of acid or alkali to solubilize them completely, adjusted to pH 7.5, and stored at 1 mg/ml in aliquots at −70°C. Purified whole *Torpedo* AChR was prepared by affinity chromatography on cobratoxin columns and eluted with carbamylcholine as previously described (12). The purified preparation was extensively dialyzed against phosphate-buffered saline (PBS) containing 0.2% cholate (no preservatives) before use in culture.

Lymphocyte lines. Lymphocyte lines were established from PBL of three patients showing positive responses to purified *Torpedo* AChR (manuscript in preparation) and from five patients showing a response to peptides. These lines were maintained in culture by restimulation at 7–10-d intervals with the relevant antigen in the presence of autologous irradiated (1,500 rads) PBL as feeder cells, followed by further expansion in recombinant human interleukin 2 (IL-2) (Boehringer, Mannheim, Federal Republic of Germany). From one of the cell lines, AChR-specific clones were obtained by limiting dilution and maintained in long-term culture by methods established for other T cell antigens (13).

Proliferation assays. Fresh lymphocytes were cultured in triplicate in round-bottomed microtiter plates (Nunclon, Gibco) at 2×10^5 cells per well (5×10^5 per well for unseparated thymus cells), in 200 μl RPMI 1640 plus 10% human A^+ serum. The T cell lines were assayed for proliferation at 2.5×10^4 cells per well in the presence of 1×10^5 irradiated (1,500 rads) autologous feeder cells. Peptides were used at dilutions ranging from 50 to 0.001 μg/ml final concentrations in the assays. Most of the patients' lymphocytes were screened against the peptides at concentrations of 50 and 20 μg/ml. Each assay plate also included triplicate wells containing 10 μg/ml *Torpedo* AChR, PHA (1 μg/ml), or cells only. Cells were cultured at 37°C in a humidified atmosphere of 95% air, 5% CO_2 for 72 h. 1 μCi per well of [^{3}H]-thymidine was then added and the plates cultured for a further 16 h when they were harvested with an Automash II Harvester (Dynatech Corp., Alexandria, VA). Thymidine incorporation was measured in a liquid scintillation counter. The stimulation index (SI) was calculated as follows: SI = (counts per minute of cells + antigen) ÷ counts per minute of cells alone. Values cited are the highest obtained and are not necessarily those at the highest antigen concentration.

HLA typing. HLA typing was carried out as previously described (14) using the 11th Histocompatibility Workshop-validated antisera in a complement-mediated microcytotoxicity test.

Results

Proliferation of PBL. The proliferation assays of PBL from the 34 myasthenic patients and 17 controls are summarised in Fig. 1. For the purposes of this study, an SI > 2.5 was regarded as a positive response. With several peptides, there was no suggestion of a response in either patients or controls, viz. 195–212, 310–327, 373–389, 422–437, and *Torpedo* 185–196. When the first three peptides in the series were used, i.e., 1–15, 84–98, and 99–116, some patients showed a small stimulation (SI = 1.5–2.5), and for peptide 1–15 there was a similar small response in two of the controls (Fig. 1).

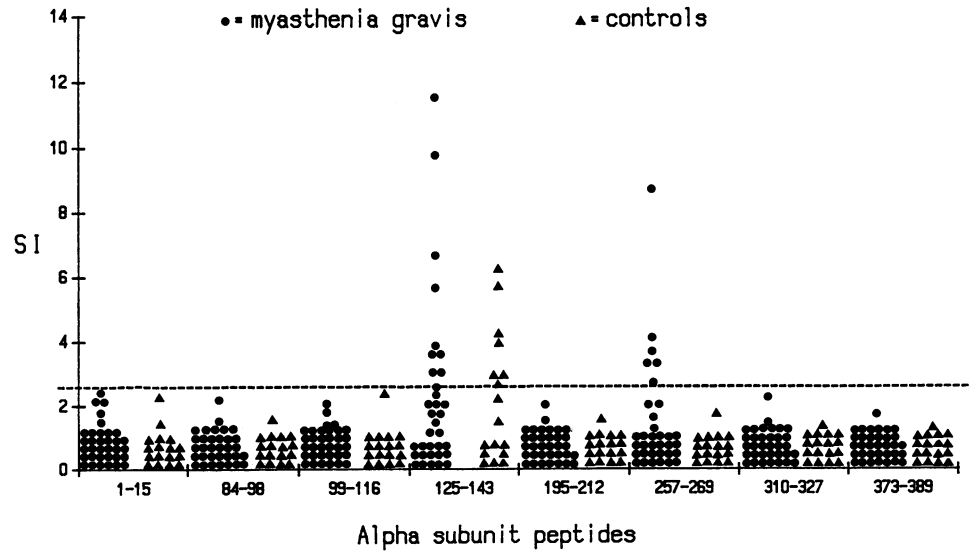

Figure 1. Proliferative responses of PBL to peptide sequences of the human AChR α-subunit. PBL of 34 MG patients and 17 controls were cultured with peptides at concentrations of 50 and 20 μg/ml. After 72 h in culture, proliferation was measured by the uptake of [^{3}H]thymidine. Results are expressed as the highest SI for each peptide.

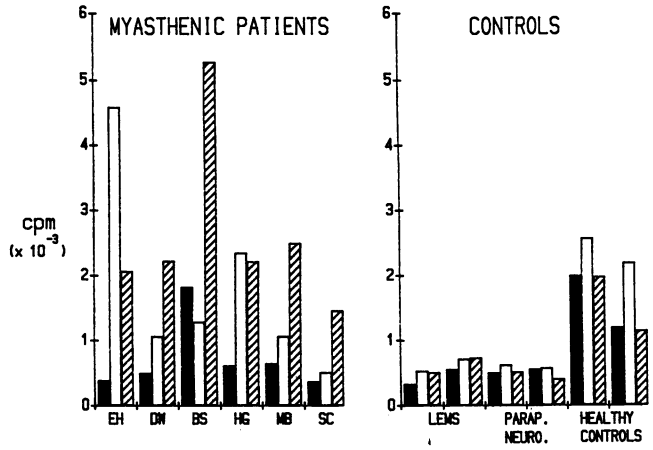

Figure 2. Proliferative responses of PBL to peptide 257–269. PBL from 6 of 34 MG patients showed a proliferative response (SI > 2.5) to peptide 257–269 at concentrations of either 50 or 20 μg/ml. (■) Cells alone; (▨) + peptide 257–269. Four of the six showed some response to *Torpedo* AChR (□), although only two had an SI > 2.5 (EH = 11; HG = 3.8). None of the 17 controls showed a response to peptide 257–269 as illustrated by results from six subjects (*right panel*). Results are expressed as mean counts per minute of triplicate cultures. Abbreviation: PARAP. NEURO., paraproteinemic neuropathy.

By contrast, peptide 257–269 induced significant proliferative responses only among MG patients, with no responses in controls (Fig. 1). In six of the patients the SI was between 2.6 and 9.0, and four of these also gave moderate responses to purified *Torpedo* AChR (Fig. 2). None of the controls gave an SI > 2.5 with this peptide as illustrated by the six examples in Fig. 2.

The remaining positive responses were largely confined to the region 125–143. In native AChR, there is a disulphide bond (S–S) between cysteines 128 and 142, reproduced in peptide 125–143. Peptide 125–143 is identical except that it has a serine at 128, and therefore no S–S bond: this is also absent in the shorter peptide 129–143. A total of nine MG patients responded to the sequence 125–143 (Fig. 3); seven of them responded to 129–143 and two of these also responded to 125–143 and 125–143 (S–S). The remaining two MG patients reacted to peptide 125–143 (S–S) alone. 5 of the remaining 25 patients gave borderline results with at least one of these peptides (Fig. 1). 7 of the 17 controls also showed positive responses to peptide 129–143 and peptide 125–143 and 125–143 (S–S) (Fig. 1). One of these was a healthy laboratory worker who also responded on the same occasion to purified *Torpedo* AChR, giving an SI of 4.0 to peptide 129–143. These results, expressed in counts per minute, from MG patients and controls are shown in Fig. 3.

Proliferation of thymic and lymph node cells. Low-density cells from only two of the six hyperplastic MG thymus samples responded to peptide 125–143 (S–S) (SI = 2.7 and 4.1) whereas PBL from these two cases were unreactive, but did respond in one of the remaining four cases. Low-density cells from one thymoma showed an SI of 2.7 and 2.8 to peptide 125–143 (S–S) and 129–143, respectively, but both the adjacent uninvolved thymus and the PBL were unresponsive to this peptide.

The three lymph node cell samples gave SIs of 23.6, 8.2, and 2.4 with peptide 125–143 (S–S), whereas the values for PBL in the same cases were 10, 2.9, and 1.4, respectively.

There was no evidence of a response to peptide 257–269 from any of the thymus and lymph node samples. Blood lymphocytes from six of these seven patients had also failed to respond to this peptide, although PBL from a thymoma patient (*MB*) had shown a response (Fig. 2).

Proliferation of lymphocyte lines. Lymphocyte lines raised against purified *Torpedo* AChR had previously been established from three patients whose PBL had shown proliferative responses (SI > 2.5–8.3) at the first test. These lines have been maintained in culture for 3 mo to 1 yr and have consistently shown a positive response to *Torpedo* AChR although this varied quantitatively from week to week. Lines were also established from PBL of patients CB and HG using peptide 125–143 (S–S).

Lines raised against *Torpedo* AChR were tested against all the peptides at concentrations ranging from 50 to 0.001 μg/ml. There was no evidence of stimulation by any of the peptides for any of the three lines tested nor from a clone (C9) derived from one of them (Fig. 4), even though responses to *Torpedo* AChR were high at the time of testing (SI = 6–10).

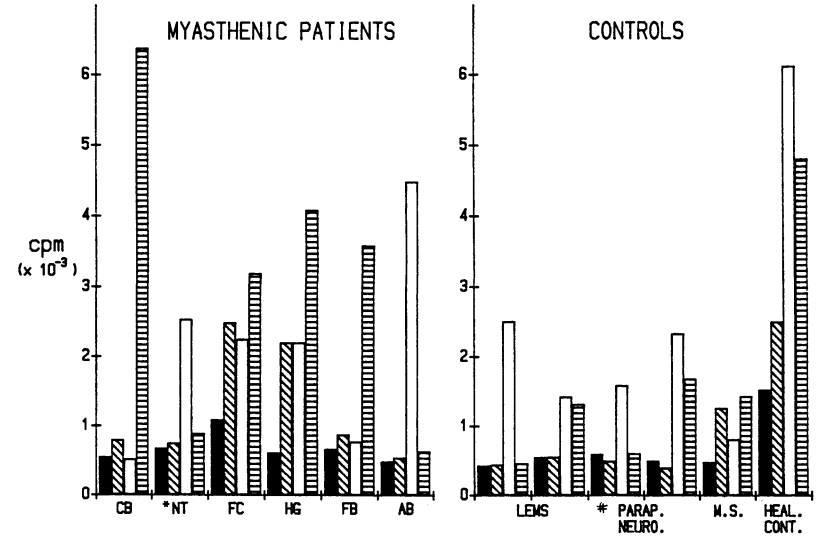

Figure 3. Proliferative responses of PBL to peptide sequences 125–143. PBL from 9 of 34 MG patients and 7 of 17 controls showed proliferative responses to peptide sequence 125–143 at concentrations of 50 and 20 μg/ml. (■) Cells alone; (▧) 125–143; (□) 129–143; (▤) 125–143, S–S. *Results from patients JC, DW, and JH showed a similar pattern to NT. *Results from one other patient with paraproteinemic neuropathy showed a similar pattern to this patient. Abbreviations: M.S., multiple sclerosis; HEAL. CONT., healthy control.

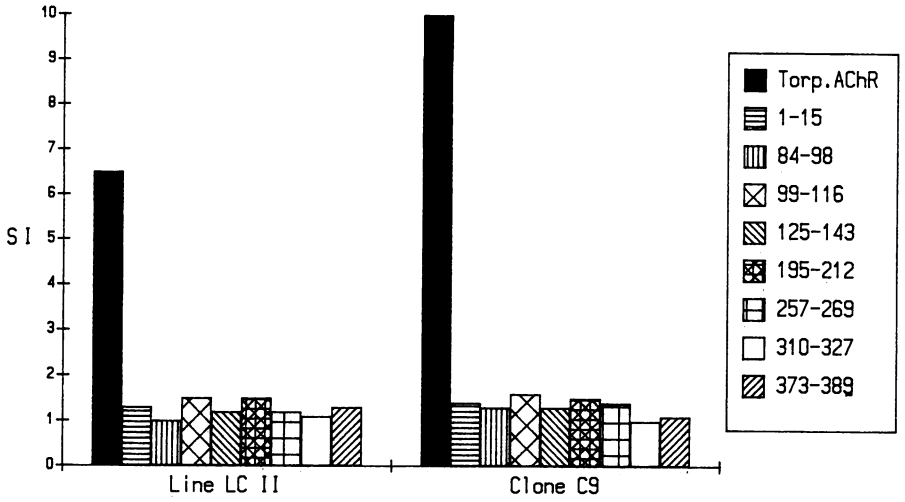

Figure 4. Proliferative response of *Torpedo* AChR-specific T cell line and clone to human AChR peptides. T cell line (LC 11) or clone (C9) cells from MG patients, raised against *Torpedo* AChR (5 μg/ml) were tested for a proliferative response to various concentrations (50–0.01 μg/ml) of human AChR peptides. Results shown in this experiment are expressed as SI obtained with peptides at concentrations of 25 μg/ml.

Lines raised against peptide 257–269 from patients EH and SC are still at an early stage: after 4–6 wk in culture the SIs for this peptide were 3.7 and 4.6 (not shown). Interestingly, reactivity to *Torpedo* AChR was concomitantly sustained (SI = 2.0 and 4.7). The lines raised against peptide 125–143 (S–S) showed a good response to the stimulating antigen on retesting (Fig. 5). Patient HG initially responded to both Torpedo AChR and peptide 125–143 but a cell line selected with the latter lost responsiveness to *Torpedo* AChR by 3 wk indicating that the populations of T cells specific for *Torpedo* AChR and human peptides were separate. Two lines raised from patient CB against peptide 125–143 (S–S) showed minimal responses to 125–143 or 129–143 initially, but the responses to 125–143 (but not 129–143) increased by day 19 (Fig. 5). The apparent fluctuations in 125–143 and 125–143 (S–S) responses may reflect trivial differences in processing rather than in T cell fine specificities. Evidently peptide 125–143 (S–S) rapidly selected cells that recognised peptides 125–143, linked or unlinked. These lines did not respond to any of the other peptides.

HLA type and peptide responsiveness. The HLA types of the majority of the patients and of the controls who responded to peptides are shown in Table II. Although there is a high overall frequency of HLA-B8 and DR3, these may be slightly underrepresented in the responders, who show a small excess of DR2 (neither being statistically significant).

Discussion

The human AChR α-chain sequence contains 22 of the recurring amino acid motifs identified by Rothbard and Taylor (8). The 11 peptides used here were selected because they include many of these sites (Table I) and closely resemble other known T cell determinants. These authors have already successfully identified T cell epitopes in influenza virus haemagglutinin, matrix and nucleoproteins (11, 15) and in ovalbumin (16). This approach has now apparently located a further site in the human AChR (257–269) that is recognized by ~ 20% of our MG cases and none of 17 controls. This peptide sequence is identical in *Torpedo* AChR, and indeed PBL responding to this peptide also responded to native *Torpedo* AChR in most cases. The responses to peptide 257–269 suggest that a previously unsuspected external region of the AChR (between two of the transmembrane domains) may be an important focus for autoreactive T cells in MG.

A second sequence (125–143), including one of the intrachain S–S bonds, also stimulates T cells in a similar proportion of patients, and had previously been suggested as an important T cell epitope in experimental MG (EAMG) in rats (2) either S–S linked or unlinked. Similarly, two patients (HG and FC) did not distinguish between the straight and S–S-looped variants of this peptide (Fig. 3). However, other cases showed a

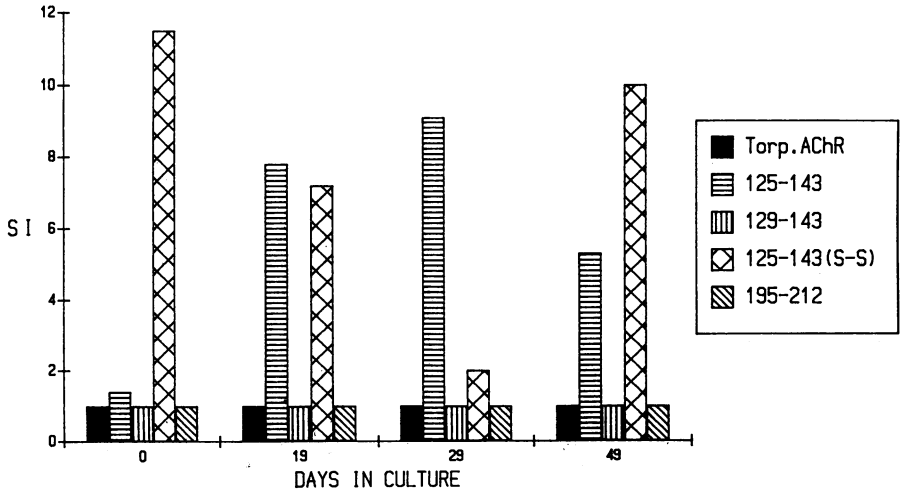

Figure 5. Proliferative response of T cell line raised against peptide 125–143 (S–S). A T cell line from PBL of MG patient (CB) raised against peptide 125–143 (S–S) was tested against the antigens illustrated after various periods of time in culture. Results at day 0 indicate the initial response of the PBL from whom this line was selected. *Torpedo* AChR was used at a concentration of 10 μg/ml and peptides at a concentration of 25 μg/ml. Results are expressed as SI.

Table II. HLA Types of Responders to AChR α-Subunit Peptides

Myasthenia gravis patients		HLA type		
Identification	MG Subgroup	A	B	DR
To peptide 257–269				
EH	Young onset	11, 28	5, 8	2, 3
DW	Young onset		Not typed	
HG	Young onset	9, 11	16, 27	1, —
BS	Young onset	1, 2	12, —	2, 7
SC	Young onset	1, 2	8, 12	3, 7
MB	Thymoma	2, —	27, 18	6, 8
To peptide 125–143				
CB	Young onset	10, 11	35, 18	1, 2
NT	Young onset	2, 9	8, 12	3, 6
DW	Young onset		Not typed	
HG	Young onset	9, 11	16, 27	1, —
JC	Young onset	2, —	7, 60	2, 4
AB	Young onset	1, —	8, 18	2, 3
FB	Young onset	1, 3	8, 35	1, 3
JH	Old onset	2, 19	7, 60	2, 5
FC	Thymoma	2, 32	12, 15	3, —
	Controls			
IP	LEMS	2, 10	18, 37	2, —
DR	LEMS	2, 25	8, 60	3, 4
MT	Cerebellar syndrome		Not typed	
DI	PN*		Not typed	
AR	PN		Not typed	
RM	PN		Not typed	
NS	Healthy	3, 9	7, 13	7, —
	Nonresponders: MG group (to either peptide)			
		B8	$DR3^+$	9/21
			$DR2^+$	12/21

* Paraproteinemic neuropathy.

modest response only to the shorter 129–143 peptide, perhaps implying that removal of residues 125–128 improves binding either to the restricting class II antigen or to the T cell receptor. Interestingly, and in contrast to peptide 257–269, there is little T cell cross-reactivity between this region and *Torpedo* AChR. Thus T cell lines selected with peptide 125–143 (S–S) failed to respond to *Torpedo* AChR, and vice versa, even though there are only two amino acid differences (human Glu → Gln at 139 in *Torpedo,* and Ser → Thr at 143). Perhaps these are critical, or possibly the carbohydrate attached at 141 affects the processing of the intact AChR so that this peptide is not presented optimally. In other systems an analogous lack of T cell cross-reactivity between native antigens and peptides from them has also been reported as with hen eggwhite lysozyme (17). Possibly, with other peptides, differences in adjacent sequences may also affect T cell recognition. Whatever the reasons, it seems clear that *Torpedo* AChR may not stimulate all the potentially autoreactive T cells in MG patients, and that the mammalian AChR fragments that are now being tested in this and other laboratories may prove to be more appropriate (18).

The responses to peptide 125–143 in controls may imply a cross-reaction with some environmental antigen (e.g., bacterial) that could be coincidental or might be involved in provoking autoreactivity in susceptible subjects. Alternatively, T cell reactivity to AChR may conceivably be more widespread than previously suspected, and these clones may be controlled more effectively in healthy subjects than in myasthenics or indeed T cells reactive to this peptide may not inevitably be pathogenic in humans.

It is interesting that peptides 1–15, 84–98, and 99–116 stimulated so poorly since the majority of autoantibodies appear to bind near this area (1, 5). Similarly, those peptides corresponding to parts of the cytoplasmic domain of the AChR also failed to stimulate T cells from any of the MG patients, including the four with thymoma, despite the possibility that this region of the AChR may be expressed by thymomatous epithelial cells (19). However, because quite small differences in peptide length or sequence can considerably alter recognition and there are substantial gaps between some of the regions studied here, negative results should be interpreted with caution.

Further studies are also required on the responses of thymic cells. The normal and MG thymus contain rare muscle-like myoid cells that express AChR both in culture (20) and in situ (21) and it has been suggested that these may somehow initiate autoreactivity in MG (20), and might be responsible for the follicular hyperplasia that is common in the MG thymus (22). It is interesting therefore that in a more extensive series, we have found that T cell responses to *Torpedo* AChR are more consistent in low-density thymus cells than with the same patients' PBL (manuscript in preparation). Melms et al. (4) have similar results. Although these initial observations support the concept of autosensitisation in the thymus, it is hard to exclude the alternative possibility that T cells originally induced in the periphery selectively home to the thymic medulla later in the response.

Some of the responders recognized *Torpedo* AChR in addition to peptides 129–143 and 257–269 (e.g., patient HG). Their AChR-reactive T cells are evidently heterogeneous, which implies that selective therapy with clonotypic antibodies to the T cell receptor may not always be straightforward.

There was no clear association detected between HLA type and the T cell epitopes recognized: if anything, there was a slight preference for DR2 rather than DR3, in spite of the very strong overall HLA-B8-DR3 bias in young-onset MG patients in the United Kingdom (Table II) (14). It has been suggested that the latter is a nonspecific predisposition (23) and if the present tentative conclusions can be confirmed, they may support this view. However, in a similar study, Brocke and his colleagues (manuscript submitted for publication) have found responses to peptide 257–269 that were apparently related to the presence of HLA-B8 and DR3 in myasthenics (and some controls) in Israel. More frequently, they found reactivity to peptide 195–212 that was confined to $DR5^+$ subjects. The MG population in Israel is clearly different from ours: perhaps the rarity of the DR5 allele in our subjects (2 of 33 MG patients) (Table II) might account for the unresponsiveness to this peptide. Further studies may identify a different restricting allele for the response to peptide 257–269 in our study.

Acknowledgments

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