T Helper Cell Recognition of Muscle Acetylcholine Receptor in Myasthenia Gravis

Epitopes on the γ and δ Subunits

Angelo A. Manfredi, Maria Pia Protti, Mark W. M. Dalton, James F. Howard, Jr.,* and Bianca M. Conti-Tronconi
*Department of Biochemistry, University of Minnesota, St. Paul, Minnesota 55108; Department of Pharmacology, University of Minnesota, Minneapolis, Minnesota 55455; and *Department of Neurology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7025*

Abstract

We tested the response of $CD4^+$ cells and/or total lymphocytes from the blood of 22 myasthenic patients and 10 healthy controls to overlapping synthetic peptides, 20 residues long, to screen the sequence of the γ and δ subunits of human muscle acetylcholine receptor (AChR). The γ subunit is part of the AChR expressed in embryonic muscle and is substituted in the AChRs of most adult muscles by an ε subunit. The δ subunit is present in both embryonic and adult AChRs. Adult extrinsic ocular muscles, which are preferentially and sometimes uniquely affected by myasthenic symptoms, and thymus, which has a still obscure but important role in the pathogenesis of myasthenia gravis, express the embryonic γ subunit. Anti-AChR $CD4^+$ responses were more easily detected after $CD8^+$ depletion. All responders recognized epitopes on both the γ and δ subunits and had severe symptoms. In four patients the $CD4^+$ cell response was tested twice, when the symptoms were severe and during a period of remission. Consistently, the response was only detectable, or larger, when the patients were severely affected. (*J. Clin. Invest.* 1993. 92:1055–1067.) Key words: embryonic acetylcholine receptor • autoimmunity • T epitopes

Introduction

In myasthenia gravis (MG)[1] an autoimmune response against muscle nicotinic acetylcholine receptor (AChR) occurs (1–4). High affinity IgG antibodies cause AChR destruction/dysfunction and myasthenic symptoms (1, 4). Their synthesis requires intervention of anti-AChR T helper (Th) $CD4^+$ cells (5, 6), which have been demonstrated in the blood and thymus of MG patients, can be propagated in vitro (5–15), and recognize AChR epitopes in a DR-restricted fashion (16).

Muscle AChRs exist in two developmentally regulated isoforms, both formed by four homologous subunits (reviewed in references 17 and 18). Embryonic AChR is formed by α, β, γ, and δ subunits. On innervation, the γ subunit is normally substituted by a homologous ε subunit to yield adult AChR, an $\alpha\beta\varepsilon\delta$ oligomer (17–20). The AChR α subunit contains the so-called main immunogenic region (MIR), i.e., the set of largely overlapping epitopes that dominates the antibody response in MG (21–23), and important constituent elements of binding sites for cholinergic ligands (reviewed in reference 24). Most studies on the sequence regions forming T cell epitopes have focused on the α subunit (10–14). However, since T and B cells tend to recognize different parts of the same protein antigen ("complementarity" of the T and B repertoire [25, 26]), other AChR subunits may form epitopes important for sensitization of pathogenetic T cells. Furthermore, MG patients frequently have antibodies specific for the embryonic AChR (27, 28), and in some cases all anti-AChR antibodies uniquely recognize embryonic AChR (29). Such antibodies must recognize epitopes formed primarily or exclusively by the γ subunit. Th cell lines specific for the embryonic AChR, and recognizing epitopes on the γ subunit, can be propagated from MG patients (30, 31).

Particular interest in the anti-AChR sensitization against the embryonic γ subunit is justified by the recent finding that extrinsic eye muscles (EOM) of adult mammals express the AChR γ subunit (32). This well explains the embryonic-like electrophysiological properties of some EOM AChRs (33, 34). The EOM are generally the first and sometimes the only muscles to manifest myasthenic weakness, suggesting that the AChRs they express have unique structural features (33, 34). The embryonic γ subunit is a likely candidate as the EOM-specific structure recognized in MG. The γ subunit is expressed in normal thymus (35), further supporting the involvement of an embryonic AChR in MG pathogenesis, since the anti-AChR sensitization might originate within the thymus (36), which in MG patients is frequently hypertrophic or contains a thymoma (37), and contains anti-AChR T and B cells (10, 38–40).

AChR-specific long-term Th cell lines can be propagated from the blood of MG patients by stimulation with pools of synthetic peptides corresponding to the sequences of the human AChR α, β, γ, and δ subunits (13–15, 30, and Moiola, L., and B. M. Conti-Tronconi, unpublished observations). However, long-term Th lines are not ideal to study the Th epitope repertoire in MG, since during their propagation in vitro selective clonal loss or enrichment may occur, resulting in a biased repertoire of the anti-AChR Th lines. Long-term Th lines specific for the AChR α subunit recognize only epitopes within four immunodominant sequence regions (14), while direct testing of unselected $CD4^+$ cells from MG patients' blood reveals several other epitopes (41). Furthermore, propagation of

The permanent address of A. A. Manfredi and M. P. Protti is Laboratorio di Immunologia, Scientific Institute H. S. Raffaele, Milano, Italy.

Address correspondence to Dr. Bianca M. Conti-Tronconi, Department of Biochemistry, University of Minnesota, 1479 Gortner Ave., St. Paul, MN 55108.

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1. *Abbreviations used in this paper:* AChR, nicotinic acetylcholine receptor; DR, HLA-DR; EOM, extrinsic eye muscles; MG, myasthenia gravis; MIR, main immunogenic region; TAChR, *Torpedo* AChR; Th, T helper.

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Th lines is time consuming and labor intensive, and very few patients can be studied by this approach (14, 15, 30).

In this study we analyzed the response of unselected peripheral blood lymphocytes and/or of $CD8^+$-depleted, $CD4^+$-enriched blood cells to overlapping synthetic peptides to screen the sequences of the human AChR γ and δ subunits. The MG patients used are the same ones in which we investigated the Th epitope repertoire on the human AChR α subunit (41). The combined results of those two studies should yield a reasonably comprehensive assessment of the epitope repertoire in MG, and the frequency among MG patients of a Th response to embryonic AChR.

Methods

Patients. Table I summarizes the salient clinical features of the 22 MG patients, whose Th repertoire on the AChR α subunit we characterized previously (41). For 14 patients, $CD8^+$-depleted, $CD4^+$-enriched T cells were isolated and used to study their in vitro response to peptide sequences (patients 3, 6–10, 12–16, 18, 19, and 21). For six of these patients (3, 6, 7, 9, 15, and 19) a similar experiment was repeated using total PBMC. In eight patients (1, 2, 4, 5, 11, 17, 20, and 22) we studied only the response of the PBMC population. Similar experiments were carried out with cells from healthy controls (four males, six females) of different HLA haplotype, 26–44 yr of age.

Peptide synthesis and characterization. We used overlapping synthetic peptides, 11–20 residues long, corresponding to the complete sequences of human muscle AChR γ (42), δ (43), and α subunits (44) which we synthesized, characterized, and described previously (12–15, 30, 41). The peptides overlapped each other by four to eight residues. They are indicated by codes that include the letter H for human, the symbol of the corresponding subunit, and two numbers indicating the position on the subunit sequence of the amino-terminal and carboxyl-terminal residues of the peptide. The Hγ or Hδ peptides correspond to the sequence segments of the human AChR γ and δ subunits indicated along the abscissa of Fig. 2.

Purification of Torpedo AChR. Native, membrane-bound *Torpedo* AChR (TAChR) was prepared from *Torpedo californica* electric tissue and characterized as described in reference 12. The specific activity of TAChR preparations (expressed as nanomoles of α-bungarotoxin binding sites per milligram of protein) was 4–7 nmol/mg of protein (pure TAChR: 7.2 nmol/mg).

$CD8^+$ T cell depletion. $CD8^+$ T cell depletion of PBMC was done using mouse anti-CD8 antibody (OKT8; Ortho Diagnostic Systems Inc., Raritan, NJ) and "paramagnetic" beads coated with goat anti-mouse Ig antibody (Advanced Magnetics, Inc., Cambridge, MA) (12). The phenotypes of PBMC and of $CD8^+$-depleted, $CD4^+$-enriched cells (referred to as $CD4^+$-enriched cells) were determined in four experiments by FACS® analysis (FACStar®, Becton Dickinson and Company, Mountain View, CA) (12). PBMC had relative amounts of T cells and of the different T cell subsets within the normal ranges. The phenotypes of the $CD4^+$-enriched population was as follows ($n = 4$): T cells ($CD3^+$), 61.4±15%; activated T cells ($CD3^+$, DR^+), 1.8±0.6%; $CD4^+$ cells, 55.5±14.3%; activated $CD4^+$ cells ($CD4^+$, DR^+) 1.6±1%; $CD8^+$ cells, 0.5±0.1%; activated $CD8^+$ cells ($CD8^+$, DR^+), 0.1±0.1%.

Microproliferation assay. We used $CD8^+$-depleted, $CD4^+$-enriched cells, or PBMC if the yield of PBMC was too low for purification of sufficient $CD4^+$-enriched cells. PBMC or $CD4^+$-enriched cells, diluted to 1×10^6/ml in RPMI 1640 (GIBCO BRL, Gaithersburg, MD) with 10% heat-inactivated AB human serum, 2 nM L-glutamine, 100 U/ml penicillin, and 50 μg/ml streptomycin (tissue culture medium) were plated in triplicate in 96-round-bottom-well plates (in duplicate if the amount of cells was low). No further addition of antigen-presenting cells was necessary for the $CD4^+$-enriched cells, because the $CD8^+$ depletion left sufficient macrophages for antigen presentation. The cells were stimulated with each of the following antigens: PHA (10 μg/ml; Wellcome London, UK), T cell growth factor (TCGF; Lymphocult; Biotest Diagnostics Corp., Denville, NJ; final concentration of IL-2, 10 U/ml), TAChR (10 μg/ml), the pools of synthetic peptides corresponding to the complete sequence of human AChR α (α pool), γ (γ pool), or δ subunit (δ pool) (0.05, 0.1, 0.5, 1, and 5 μg of each peptide/ml), and the individual synthetic peptides present in the γ and δ pools (10 μg/ml). The pool of synthetic peptides corresponding to the complete sequence of human AChR α, γ, and δ subunits were used to determine the antisubunit response of MG patients and healthy controls. The individual synthetic peptides were used to determine the sequence segments containing epitopes recognized by the $CD8^+$-depleted, $CD4^+$-enriched population, the PBMC, or both. Blanks were wells with cells that did not receive any stimulus, and wells with cells plus a 19-residue synthetic peptide unrelated to AChR (residues 1–19 of the major intrinsic protein of bovine lens [45], 10 μg/ml); the values obtained with the two different blanks were consistently comparable. Whenever possible, more than triplicates of the unstimulated blanks were seeded to better assess the culture-to-culture variability of [^{3}H]thymidine incorporation of unstimulated cells. After 5 d the cultures were pulsed for 16 h with [^{3}H]thymidine (1 μCi per well; specific activity, 6.7 Ci/mmol; Amersham Corp., Arlington Heights, IL). Cells were collected with a Titertek multiple harvester (Skatron, Inc., Sterling, VA) and [^{3}H]thymidine incorporation was measured by liquid scintillation.

Search for sequence similarities between AChR sequence segments and unrelated proteins. We used the program FASTDB (version 5.4) (46) of the Intelligenetics suite (IG Inc., Mountain View, CA) to search for sequence similarities between the protein sequences in the databases PIR 29 and Swiss-Prot 19, and segments of the AChR α, γ, and δ subunits found to contain T epitopes (26 and this study: α19-34, α32-51, α48-67, α101-120, α118-137, α151-168, α191-207, α293-308, α304-322, α320-337, α387-405, α403-421, α419-437, γ15-34, γ30-49, γ60-79, γ75-94, γ90-109, γ105-124, γ135-154, γ180-202, γ203-222, γ218-237, γ233-252, γ248-267, γ263-273, γ269-288, γ284-303, γ297-312, γ306-325, γ321-340, γ351-370, γ366-385, γ381-400, γ411-430, γ476-495, δ1-20, δ61-80, δ91-110, δ106-125, δ121-140, δ151-170, δ196-215, δ213-230, δ226-245, δ241-260, δ256-275, δ271-290, δ346-362, δ363-386, δ373-392, δ446-465, δ461-480, δ476-496), or control AChR sequence regions that in our studies (this study and references 15 and 26) were never recognized by $CD4^+$ T cells from MG patients (α63-80, α76-93, α89-105, α135-154, α203-218, α214-234, α230-249, γ1-20, γ120-139, γ150-169, γ165-184, γ190-209, γ336-355, γ396-415, γ438-457, γ470-489, δ31-50, δ76-95, δ136-155, δ166-185, δ301-320, δ331-350, δ401-420, δ416-435, δ431-450).

Results

Response of PBMC and $CD4^+$ enriched cells to TAChR and to peptide pools

$CD4^+$-enriched cells from 7 of the 14 patients tested responded to both the γ and the δ pools (Fig. 1). When we used PBMC, only patients 11 and 20 (out of the 14 patients for which the response of PBMC was tested, see Methods) responded to the peptide pools (Fig. 1). Fig. 1 also reports the response of the same patients to the α pool. Several patients responded to the three pools to comparable extents (10, 12, 14, and 21). Other patients did not recognize one pool (the γ pool in patient 7, the α pool in patient 14, and the δ pool in patient 20). It is conceivable that, even in the absence of a $CD4^+$ response to epitopes on a given AChR subunit, the anti-AChR antibodies of that patient may still recognize epitopes formed by residues within that subunit: this is particularly likely for the α subunit, whose corresponding α pool was not recognized by patient 14, since the α subunit contains the MIR, the set of largely overlapping epitopes which dominates the antibody response in MG (21–23). However, given the conformation dependence of most

Table I. Characteristics of the Myasthenic Patients

A. Donors of $CD8^+$-depleted cells

Patient	Experiment	Age, sex	DR*	Disease class‡	Clinical course	anti-AChR Abs§	Thymectomy	I.S.
						nM		
Responders								
3	1	35, F	1, 7	4	Worsening	2.44	Y	Y
	2			2	Improved after PLEX	0.51		Y
7		27, F	w17(3), 4	2	Worsening	0	N	Y
10	1	24, F	9, w8	4	Worsening	10.2	N	N
	2			4	Improvement after PLEX	(10.2)		N
12		41, F	2, 6	4	Worsening	47	Y	Y
13		21, F	w10, w12(5)	4	General weakness	38.4	Y	Y
14	1	25, F	5	4	Worsening, some	0.06	Y	Y
21	2	27, M	w11(5)	4	Acute worsening since	0.58	Y	Y
Nonresponders								
6		87, M	1, 4	2	Static, mild	(1.81)	N	Y
8		77, M	8	4	Worsening	3.27	N	N
9		43, F	ND	2	Stable, mild weakness	(84.4)	Y	Y
14	2	25, F	5	2	Stable, moderate weakness	(0.06)	Y	Y
15		23, F	w13(6), w11(5)	2	Stable, mild weakness	(4.49)	Y	Y
16		75, M	6	IR	Static. minimal weakness	(4.73)	N	Y
18		74, M	2, 7	IR	Static, minimal weakness	(3.55)	N	Y
19		46, F	w15(2), 4	2	Improvement with I.S.	(1.15)	Y	Y
21	1	27, M	w11(5)	2	Stable, moderate weakness	(0.48)	Y	Y

B. Donors of PBMC

Patient	Experiment	Age, sex	DR*	Disease class‡	Clinical course	anti-AChR Abs (nM)§	Thymectomy	I.S.
Responders								
11		72, M	4	4	Moderate generalized,	0.8	N	Y
20		52, M	2, 7	2	Recent worsening	ND	Y	Y
Nonresponders								
1		27, M	3	4	Recent worsening	5.78	Y	N
2		74, M	ND	2	Recent worsening	1.59	N	Y
3		35, F	1, 7	2	Worsening	1.75	Y	Y
4		69, M	5, 7	2	Minimal weakness,	1.07	N	Y
5		54, M	ND	4	Progressive weakness	0.33	N	N
6		87, M	1, 4	2	Static, mild	(1.81)	N	Y
7		60, M	w17(3), 4	2	Recent worsening	0	N	Y
9		43, F	ND	2	Progressive fatigue	84.4	Y	Y
15		23, F	w11(5), w13(6)	2	Stable, mild	(4.49)	Y	Y
17		37, F	3, 4	4	Slow worsening	81.7	N	Y
19		46, F	w11(5)	4	Worsening	2.76	Y	Y
22		30, F	W17(3)	4	Static, moderate General weakness	9.46	N	Y

ND, not determined; QD, daily; PLEX, plasma exchange; I.S., immunosuppressive treatment; I.R., in remission. * Determined by standard lymphocytotoxicity (1, 3, 4, 6, 8, 11, 12, 14, 16, 17, 18, and 20) as described in reference 78 or by RFLP and oligonucleotide hybridization (7, 10, 13, 15, 19, 21, and 22) as described in reference 79. ‡ Classified as described in reference 80. § The anti-AChR antibody titers, measured as described in reference 81, were usually evaluated on serum collected the day of the experiment. When in brackets, the titers were determined within 1 yr from the day of the experiment.

anti-AChR antibodies in MG (1), in general synthetic AChR sequences cannot be used to detect binding of antibodies against native AChR, and therefore we could not address this issue using the synthetic peptide approach.

Four patients (Table II) were tested twice, when their clinical condition was deteriorating and when it was improving (3, 10, 14), or when the clinical situation had not yet deteriorated (21). Patients 3, 14, and 21 responded to the peptide pools only when they were severely affected. Patient 10 responded to all three peptide pools in both experiments.

Most patients that responded to the peptide pools responded vigorously to TAChR (Fig. 1). Exceptions were patients 7, 10 (experiment 2), 21, and 11. They may recognize epitopes not conserved in human and *Torpedo* AChRs.

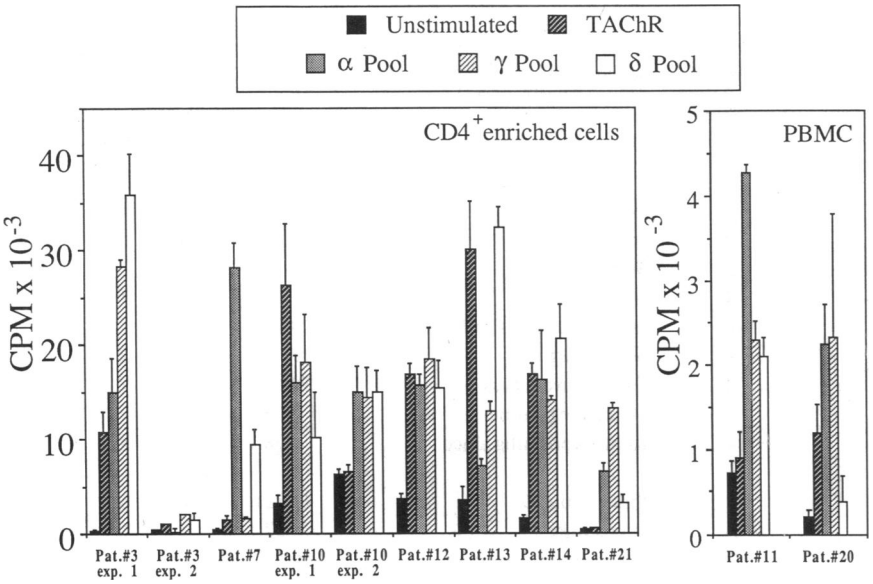


Figure 1. Response to different stimuli of CD4$^+$-enriched cells or PBMC from the nine MG patients that responded to synthetic AChR antigens, measured by microproliferation assay. The incorporation of [^{3}H]thymidine in the absence of any stimulation, or in the presence of 5 μg/ml α pool, an optimal stimulatory concentration (0.5–5 μg/ml of each peptide) of γ and δ pools, and purified TAChR (10 μg/ml) is reported. The cells always responded vigorously to PHA (10 μg/ml) and IL-2 (10 U/ml) (not reported). See text for experimental details.

The rate of cell proliferation of unstimulated PBMC or CD4$^+$-enriched cells was relatively low (< 1,600 cpm), with the exceptions of patients 10, 12, and 13 (Fig. 1 and Tables III and IV). These patients had the highest level of anti-AChR antibody (10.2, 47, and 38.4 nM, respectively; see Table I). The high basal rate of proliferation of their lymphocytes may be due to the presence of activated anti-AChR B cells. Cells from controls did not respond to TAChR or to peptide pools.

Response of PBMC and CD4$^+$-enriched cells from MG patients to individual synthetic sequences of the human γ and δ subunits

The individual peptides forming the γ and δ pools were used in microproliferation assays to detect epitope-specific response of CD4$^+$-enriched cells or PBMC. Cells from controls never responded to any individual peptides. Tables III and IV report the peptide sequences recognized by each patient. Boldface, boxed values indicate statistically significant responses ($P < 0.005$ or $P < 0.001$ as assessed by a t test). The relatively high scattering of the data may be due to the long times (~ 24 h) that elapsed between blood collection and the experiment, and to the low frequency of precursors specific for a given AChR epitope. Experiments assessing the frequency of anti-AChR T cells in the blood of MG patients reported an average frequency of 1:30,000 or less (47, 48). Even assuming that this sequence can be significantly higher in the severely affected patients that responded to the AChR epitopes, since each patients recognizes several epitopes on the AChR molecule, the frequency of T cells specific for a given T epitope is probably low enough to be a source of inhomogeneous distribution of epitope-specific CD4$^+$ cells among microcultures containing 10^5 total cells.

Response of PBMC. Only one (11) of the 14 patients whose PBMC were tested clearly recognized individual peptide sequences, in agreement with his good response to both the γ and δ pools (Fig. 1). Patient 11 recognized eight peptide sequences from the γ subunit and two from the δ subunit (Tables III and IV), which were not in homologous regions of the γ and δ subunits. Patient 20, whose PBMC had a marginal response to the γ pool and none to the δ pool, did not delectably recognize any peptide sequence.

Response of CD4$^+$-enriched cells. 8 of the 14 patients tested recognized individual synthetic sequences (Tables III and IV). The PBMC from responder patients 3 and 7 were also tested, and they did not respond to any individual peptide or peptide pool.

All CD4$^+$ responders had severe symptoms and they were mostly young women, while all but one CD4$^+$ nonresponder were improving or had mild, stable symptoms (Table I). To verify the correlation between severity of the symptoms and CD4$^+$ response to AChR sequences, four responders (3, 10, 14, and 21; see Table II) were tested twice, when they had severe or worsening symptoms and when they had mild, stable symptoms. Patients 14 and 21 recognized AChR sequences only when they were severely affected (Tables II–IV). The other two patients recognized several peptide sequences when severely affected, and only one or two peptides after recovery (Tables II–IV).

Similarities of AChR α, γ, and δ subunit sequences with unrelated proteins

Several AChR peptides recognized by CD4$^+$ cells on the α (41), γ, and δ subunits, and several control AChR sequence segments, had strong similarities with sequence regions of unrelated proteins of microbial origin. They are summarized in Table V, where the α, γ, and δ peptides are aligned on the unrelated, similar sequences. Identical or conservatively substituted residues are indicated with boxed, boldface characters. The sequence pairs containing segments of 9–16 residues with 75% or more residues identical or conservatively substituted are reported. Insertions/deletions introduced to optimize the alignment were counted as nonconservative substitutions. A length of 9–16 residues was chosen because of the length of peptides found to bind MHC molecules: eight or nine residues for MHC class I presented peptides (49–52), and somewhat longer sequences for MHC class II presented peptides (53, 54).

Discussion

In this study we identified several 20-residue sequence segments of the human AChR γ and δ subunits forming epitopes

Table II. The Response of CD4⁺ Cells to AChR Epitopes Is Related to the Clinical Status of the Patients

Patient	Experiment	Clinical status	Basal*	γ pool*	δ pool*	α pool*	γ peptides	δ peptides
3	1	Worsening	251±173	28,326±749	35,814±4,393	14,990±3,648	γ248-267	δ61-80
							γ263-273	δ91-110
							γ366-385	δ106-125
							γ411-430	δ121-140
								δ151-170
								δ213-230
								δ226-245
								δ256-275
								δ271-290
								δ346-362
								363-386
	2	Improving	466±31	1,985±41	1,423±48	432±178	None	δ91-110
10	1	Worsening	3,237±789	18,204±4,990	10,115±4,795	14,944±2,716	γ75-94	δ461-480
								δ476-496
	2	Improving	6,240±624	18,484±3,306	14,934±2,409	14,944±2,716	γ75-94	δ91-110
14	1	Worsening	1,557±303	14,095±362	20,661±1,090	16,270±5,202	γ60-79	δ196-215
							γ75-94	δ213-230
							γ269-288	δ226-245
							γ476-495	δ256-275
								δ271-290
								δ373-392
								δ461-480
								δ476-496
	2	Stable	236±24	801±203	463±140	773±76	None	None
21	1	Stable	101±32	236±168	141±33	49±18	None	None
	2	Acute worsening	464±120	13,233±588	3,175±945	6,504±861	γ75-94	δ91-110
							γ381-400	δ196-215
								δ241-260

* The values are averages±SD of the [³H]thymidine incorporation of triplicate microproliferation cultures in the presence of the indicated AChR synthetic antigens. Basal refers to the [³H]thymidine incorporation of unstimulated microcultures. See text for experimental details.

recognized by autoimmune Th cells of MG patients, and we demonstrated that sensitization against the embryonic γ subunit, which is expressed in the thymus (35) and in EOM (32), frequently occurs in MG. Although each patient had an individual pattern of response to the synthetic sequences, some regions of the γ and δ subunits were recognized by several patients of different DR type, confirming that immunodominant regions for Th sensitization exist in human AChR (14, 31, 41).

The peptides used overlapped by approximately five residues. Although we may have missed epitopes not represented by our peptides (e.g., split between peptides, or contained in a peptide that folds into a secondary structure incompatible with HLA-DR binding, or due to negative effects exerted by flanking residues [55, 56]), we should still detect a significant part of the T epitope repertoire.

Whenever possible we used a cell population depleted of $CD8^+$ cells because these cells inhibit the in vitro response of autoimmune $CD4^+$ T cells to AChR epitopes (12). An inhibitory influence of $CD8^+$ cells was confirmed by this study because the PBMC of most patients did not respond to any AChR antigen, regardless of their clinical conditions. Further, the $CD4^+$-enriched cells from two patients (3 and 7) responded well to γ peptides, δ peptides, or both (Tables III and IV), while their PBMC did not respond to any AChR peptide (not reported).

All $CD4^+$ responder patients had severe symptoms and many were young women (Table I). A correlation between $CD4^+$ response to AChR epitopes and MG severity was confirmed by testing four patients at times of different disease severity; improvement of the symptoms always coincided with reduction or disappearance of the $CD4^+$ reactivity to AChR peptides (Table II). These findings agree with previous studies which suggested a correlation between $CD4^+$ response to the AChR α subunit and symptom severity (12, 41). No correlation existed between lymphocyte response to AChR sequences and anti-AChR antibody titer, in agreement with previous results on the $CD4^+$ T cell response to AChR α subunit sequences (12, 41). The responders included two patients with high antibody titer (12 and 13, with 47 and 48 nmol, respectively), but the patients with the highest titers of antibody to the AChR (9 and 17) did not respond to γ and δ subunit epitopes at the T cell level. Several patients with little or no detectable anti-AChR antibody (7, 14, 3 [experiment 2], 21 [experiment 2], and 11) had detectable T cell responses. Since there is a poor correlation between antibody titer and disease severity, and the total anti-AChR antibody titer does not give clues to

Table III. Proliferative Response of Myasthenic Patients to Peptide Sequences from Human Muscle AChR γ Subunit

Sequence	Patient 3 Experiment 1*	Patient 7	Patient 10 Experiment 1*	Patient 10 Experiment 2*	Patient 11‡	Patient 12*	Patient 13*	Patient 14*	Patient 21*
γ15–34	NS	NS	NS	NS	NS	**13,400 ± 3,729§**	NS	NS	NS
γ30–49	NS	NS	NS	NS	**2,858 ± 621§**	NS	NS	NS	NS
γ60–79	NS	NS	NS	NS	NS	NS	NS	**5,018 ± 82§**	NS
γ75–94	NS	NS	**17,200 ± 1,572§**	**12,726 ± 2,646§**	**7,829 ± 84§**	**15,585 ± 490§**	NS	**5,293 ± 807§**	**2,684 ± 670§**
γ90–109	NS	NS	NS	NS	NS	**11,130 ± 1,918§**	NS	NS	NS
γ105–124	NS	NS	NS	NS	**3,696 ± 610§**	**14,396 ± 459§**	NS	NS	NS
γ135–154	NS	NS	NS	NS	**3,395 ± 668§**	NS	NS	NS	NS
γ180–202	NS	NS	NS	NS	NS	**14,704 ± 111§**	**11,453 ± 2,259§**	NS	NS
γ203–222	NS	**5,596 ± 4,783§**	NS	NS	NS	NS	NS	NS	NS
γ218–237	NS	**3,474 ± 3,476§**	NS	NS	NS	**18,247 ± 193§**	NS	NS	NS
γ233–252	NS	NS	NS	NS	**2,756 ± 481§**	NS	NS	NS	NS
γ248–267	**9,898 ± 1,561§**	NS	NS	NS	NS	NS	NS	NS	NS
γ263–273	**6,320 ± 2,464§**	NS	NS	NS	**3,157 ± 350§**	NS	NS	NS	NS
γ269–288	NS	NS	NS	NS	NS	NS	NS	**7,885 ± 2,275‖**	NS
γ284–203	NS	**6,127 ± 4,112§**	NS	NS	NS	NS	NS	NS	NS
γ297–312	NS	NS	NS	NS	**2,778 ± 508§**	NS	NS	NS	NS
γ306–325	NS	NS	NS	NS	NS	**13,329 ± 1,430§**	NS	NS	NS
γ321–340	NS	NS	NS	NS	**4,914 ± 244§**	**14,129 ± 1,993§**	NS	NS	NS
γ351–370	NS	NS	NS	NS	NS	NS	**12,162 ± 1,744§**	NS	NS
γ366–385	**6,534 ± 1,117§**	NS	NS	NS	NS	**9,340 ± 220§**	NS	NS	NS
γ381–400	NS	NS	NS	NS	NS	NS	NS	NS	**5,267 ± 2,040§**
γ411–430	**5,165 ± 1,464§**	NS	NS	NS	NS	**16,563 ± 795§**	NS	NS	NS
γ476–495	NS	NS	NS	NS	NS	NS	NS	**7,941 ± 2,625§**	NS
Blank	793 ± 1,418 (*n* = 15)	1,128 ± 857 (*n* = 21)	6,261 ± 4,485 (*n* = 20)	6,109 ± 2,248 (*n* = 9)	713 ± 147 (*n* = 3)	3,636 ± 546 (*n* = 3)	3,871 ± 2,707 (*n* = 9)	1,556 ± 303 (*n* = 3)	576 ± 153 (*n* = 6)

Results are means of triplicate cultures (or, for the unstimulated blanks, of as many replicate cultures as indicated in parentheses) ± SD. Boldface, boxed numbers are significantly higher than the unstimulated controls (determined by *t* test. § $P < 0.001$; ‖ $P < 0.005$; NS, not significant).

* $CD4^+$-enriched cells were used.

‡ PBMC were used.

Table I V. Proliferative Response of Myasthenic Patients to Peptide Sequences from Human Muscle AChR δ Subunit

Sequence	Patient 3 Experiment 1*	Patient 3 Experiment 2*	Patient 7*	Patient 10 Experiment 1*	Patient 10 Experiment 2*	Patient 11‡	Patient 12*	Patient 13*	Patient 14*	Patient 21*
δ1–20	NS	NS	NS	NS	NS	NS	**12,801 ± 650**§	NS	NS	NS
δ61–80	**10,207 ± 61**§	NS	NS	NS	NS	NS	NS	NS	NS	NS
δ91–110	**11,365 ± 2,574**§	**2,949 ± 888**§	NS	NS	**14,970 ± 1,905**§	**2,115 ± 34**§	**10,265 ± 3,361**‖	NS	NS	**1,527 ± 178**§
δ106–125	**7,786 ± 2,250**§	NS	NS	NS	NS	NS	NS	NS	NS	NS
δ121–140	**11,087 ± 5,548**§	NS	NS	NS	NS	NS	NS	**10,588 ± 1,904**‖	NS	NS
δ151–170	**15,189 ± 3,464**§	NS	NS	NS	NS	NS	NS	NS	NS	NS
δ196–215	NS	NS	NS	NS	NS	NS	NS	NS	**9,679 ± 7,821**§	**1,539 ± 133**§
δ213–230	**5,460 ± 2,599**§	NS	NS	NS	NS	**2,163 ± 292**‖	**13,456 ± 398**§	NS	**4,206 ± 1,130**§	NS
δ226–245	**26,647 ± 10,159**§	NS	NS	NS	NS	NS	NS	NS	**4,939 ± 3,149**§	NS
δ241–260	NS	NS	NS	NS	NS	NS	NS	NS	NS	**1,363 ± 501**§
δ256–275	**25,693 ± 4,961**§	NS	NS	NS	NS	NS	**12,313 ± 6,774**§	NS	**2,426 ± 1,194**‖	NS
δ271–290	**12,264 ± 3,383**§	NS	**3,135 ± 487**§	NS	NS	NS	**16,909 ± 10,706**§	**13,277 ± 2,182**§	**8,614 ± 6,628**§	NS
δ346–362	**16,131 ± 7,221**§	NS	NS	NS	NS	NS	**14,201 ± 4,779**§	NS	NS	NS
δ363–386	**12,829 ± 2,462**§	NS	NS	NS	NS	NS	**11,668 ± 6,476**‖	NS	NS	NS
δ373–392	NS	NS	NS	NS	NS	NS	**14,741 ± 6,065**§	NS	**5,444 ± 718**§	NS
δ446–645	NS	NS	NS	NS	NS	NS	**17,783 ± 9,914**§	NS	NS	NS
δ461–480	NS	NS	NS	**22,313 ± 1,204**§	NS	NS	NS	NS	**4,494 ± 2,202**§	NS
δ476–496	NS	NS	NS	**15,941 ± 5,009**‖	NS	NS	NS	**11,831 ± 502**§	**8,107 ± 1,846**§	NS
Blank	793 ± 1,417 (n = 15)	590 ± 458 (n = 27)	1,128 ± 857 (n = 21)	6,261 ± 4,485 (n = 20)	6,109 ± 2,248 (n = 9)	713 ± 147 (n = 3)	4,722 ± 2,831 (n = 23)	3,871 ± 2,787 (n = 9)	1,623 ± 1,126 (n = 24)	576 ± 143 (n = 6)

Results are means of triplicate (occasionally duplicate) cultures ± SD. Boldface, boxed numbers are significantly higher than the unstimulated controls (as determined by t test; § $P < 0.001$; ‖ $P < 0.005$. NS, not significant). See Methods for experimental details.

* $CD4^+$-enriched cells were used for the microproliferation assay.

‡ PBMC were used for the microproliferation assay.

Table V. Similarities with Microbial Proteins of Human AChR Sequence Regions Forming T Epitopes, and of Control AChR Sequences

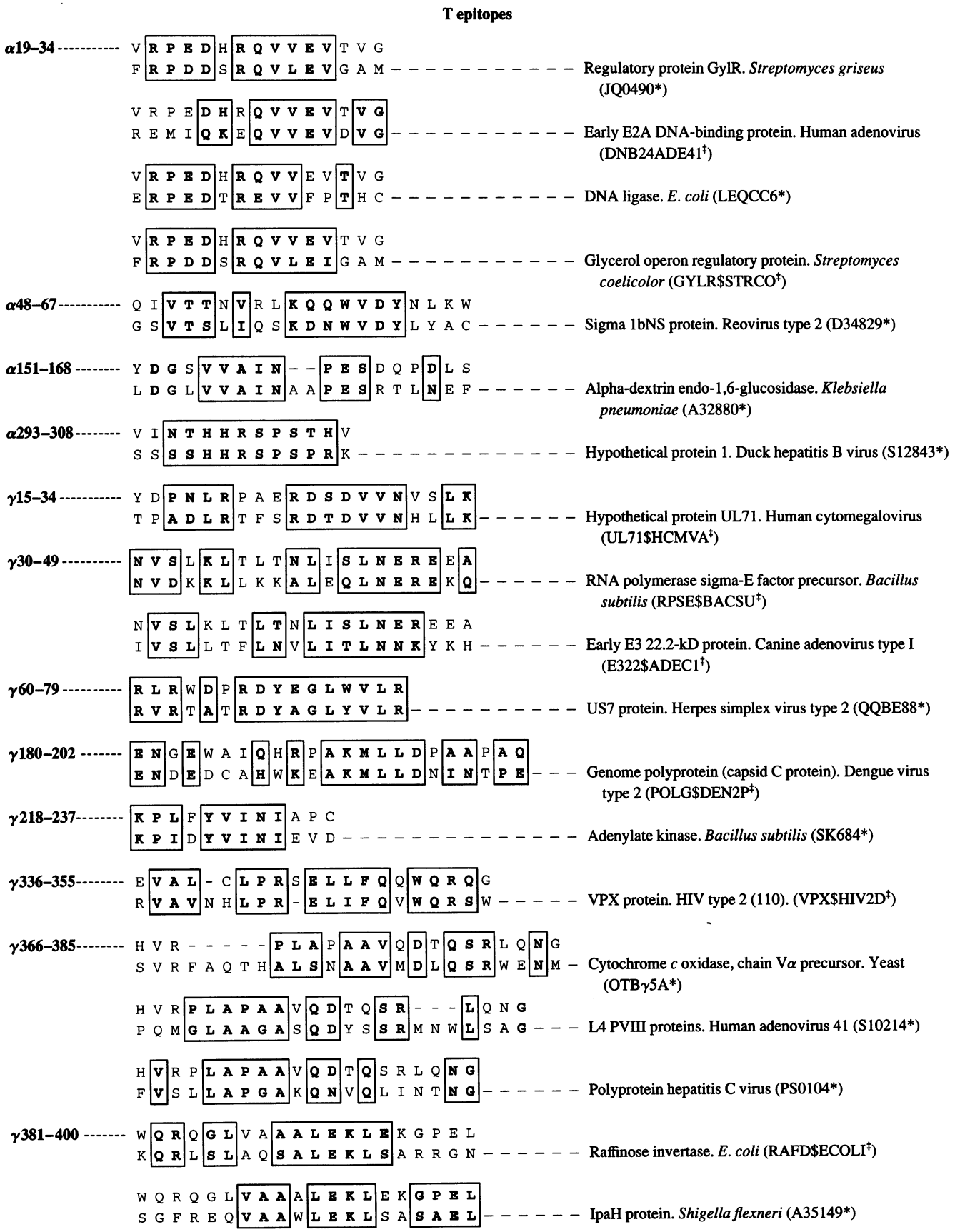
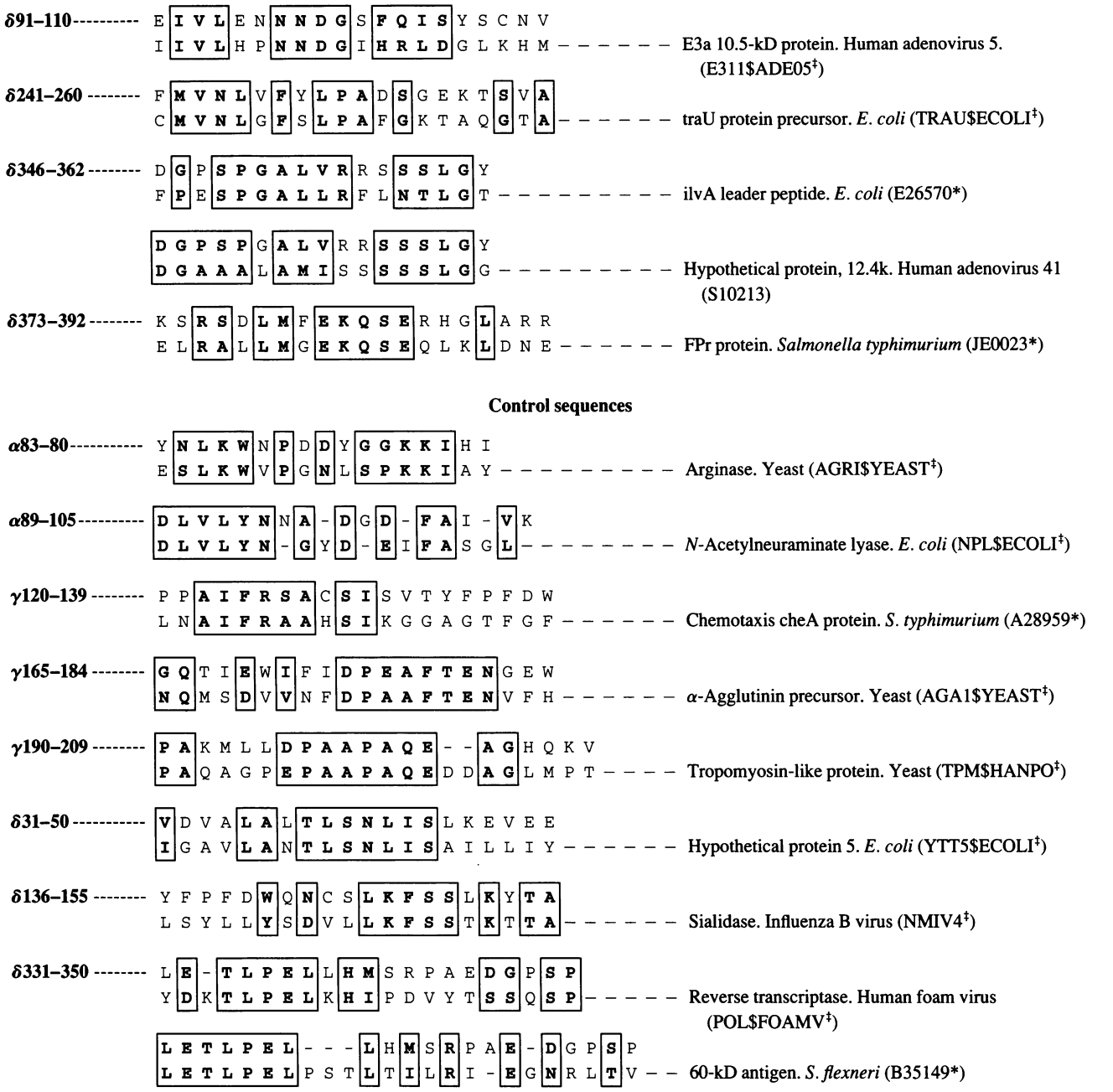


Table V. (Continued)



For 44 sequence regions of the human AChR α, γ and δ subunits identified in this or in previous studies (14, 15, 31) as forming epitopes for CD4+ cells, and for 26 control AChR sequence segments corresponding to synthetic peptides never recognized by CD4+ cells from MG patients, we searched for similarities with the sequence of the proteins present in the data bases PIR 29 and Swiss-Prot 19. The table reports only the sequence regions for which similarities with unrelated microbial proteins were found. See text for further details.

* From PIR 29.

‡ From Swiss-Prot 19.

the involvement of the different AChR subunits in the anti-AChR antibody response, it is impossible to reach any conclusions on the complex relationships between the extent of T cell sensitization, antibody synthesis by the AChR specific B cells, and severity of the symptoms. The finding that the two antibody-negative patients (7 and 14) had T cell sensitization to the AChR suggests that at least in some cases of antibody-negative MG an immune anti-AChR response occurs.

Table VI summarizes the response of CD4+-enriched cells and PBMC from MG patients to the γ and δ peptides, and to similar peptides corresponding to the α subunit sequence (41). Although all responders recognized one or more epitopes on all three AChR subunits, including the embryonic γ subunit, the relative contribution of each subunit to the repertoire of anti-AChR Th cells was characteristic for each patient. Therefore, several AChR epitopes are involved in Th sensitization in any

Table VI. Response of CD4⁺-enriched Cells and PBMC to Synthetic Human AChR Sequences

Patient (experiment No.)	α pool	γ pool	δ pool	α peptides*	γ peptides	δ peptides
3 (1)	++	++	+++	+++++	++++	++++++++++
3 (2)	−	−	−	+	−	+
7	+++	−	+	+	+++	+
10 (1)	++	++	+	+++	+	++
10 (2)	+	+	+	+	+	+
11‡	++	+	+	+++	++++++++	++
12	++	++	++	++++++	++++++++++	+++++++++
13	+	+	+	++	++	+++
14	++	++	+	+++	++++	++++++++
21 (2)	+	+	+	+	++	+++

Notice that while for responses to the peptide pools the number of pluses indicates the extent of the response, for responses to the individual synthetic peptides (columns labeled α peptides, γ peptides, and δ peptides) the number of pluses refers to the number of peptides recognized within each AChR subunit by the patients. ‡ PBMC were used; otherwise CD4⁺-enriched cells were used. * The results for the α pool and the α peptides are from reference 41.

MG patient, and Th sensitization against the embryonic γ subunit occurs frequently in MG, in agreement with easy propagation of CD4⁺ cell lines specific for embryonic AChR (30, 31). Recognition of γ subunit sequences is not due to cross-reactivity with homologous regions of other subunits, because comparison of the sequence segments recognized by the same patient on the γ and δ subunits (Tables III and IV) clearly indicates that different subunit regions form epitopes for the same patient. Comparison of the γ with the δ subunit is particularly meaningful, because their similarity is much higher than with α and β subunits (19, 20).

Even if the anti-AChR sensitization involves an embryonic-like AChR within the thymus, Th cells directed against the α, β, and δ subunits, shared by embryonic and adult AChRs, will be preferentially stimulated and expanded by the relatively abundant muscle AChR. Anti-γ subunit Th cells may eventually become a small minority. Still, their existence in many MG patients points to an origin of the anti-AChR response in a tissue other than skeletal muscle, and possibly within the thymus. The beneficial effects of thymectomy on MG symptoms (36, 37) may be due to removal of a reservoir of embryonic AChR in a tissue rich in antigen-presenting cells.

The relatively large number of epitopes recognized by any patient indicates that at least during the later phases of the anti-AChR response, when myasthenic symptoms become obvious, the whole AChR molecule is involved in the autoimmune response, but it does not exclude that the anti-AChR sensitization may be initiated by an altered form of the AChR, as occurs in experimental systems (57), or by mechanisms of molecular mimicry between a small region on the AChR molecule and a viral or bacterial epitope: once tolerance to a multideterminant antigen is broken for one epitope as result of molecular mimicry or altered self proteins, responsiveness to multiple epitopes may develop (58). We therefore searched for sequence similarities between sequence segments of the AChR forming T epitopes and unrelated proteins. We did similar searches for control AChR sequences, i.e., sequence regions of the α, γ, and δ subunits never recognized by the MG patients we studied (14, 15, 31, 41). We cannot exclude that some of these control sequences may form T epitopes for other MG patients. Although several T epitope and control sequences resembled sequence regions of other proteins, including proteins of common human pathogens (Table V), our findings do not allow conclusions about the role of molecular mimicry in autoimmune responses, because similarities with microbial proteins were not uniquely found for T epitopes, and even small sequence differences may drastically modify or abrogate the T cell recognition of an epitope sequence (59, 60). Potential immune cross-reactivity between unrelated microbial and mammalian proteins may be relatively frequent because certain short amino acid sequences (three to five residues), perhaps corresponding to specific three-dimensional motifs, occur with higher frequency than statistically expected, making it likely that fragments of alien proteins long enough for T epitope formation (49–54) may share substantial similarities with self protein fragments (61, 62). A complete purging from the T repertoire of all potentially autoreactive T cells would severely limit the T repertoire and impair the ability to respond against pathogens. Further filters must exist in the immune system to avoid widespread occurrence of autoimmune phenomena: for example, the necessity for an alien sequence, resembling a self protein, to be processed and presented, and the availability of potentially autoreactive T clones.

Three control sequences (γ165-184, γ190-209, and δ31-50) share virtually identical stretches of seven to eight residues with microbial proteins from common human pathogens/guests (yeast, *Escherichia coli,* influenza virus). It is conceivable that the use of biosynthetic or synthetic AChR sequences inclusive of these regions may propagate corresponding T clones from healthy controls and give the misleading impression of a high frequency of anti-AChR Th cells in healthy humans.

The data in Fig. 2 summarize the location on the γ and δ subunit sequences of the peptide epitopes identified in this study (*hatched columns*) and those identified previously (15, 31) by the use of anti-AChR T cell lines (*black columns*). Many epitopes are present on both the γ and the δ subunits. On the γ subunit, two sequence regions are recognized by several patients (the overlapping peptides γ60-79/γ75-94 and γ321-340). Also along the δ subunit, four relatively immunodominant sequence regions seem to exist (δ91-110, δ196-215/δ213-230, δ256-275/δ271-290, and δ461-480/δ476-496), recog-

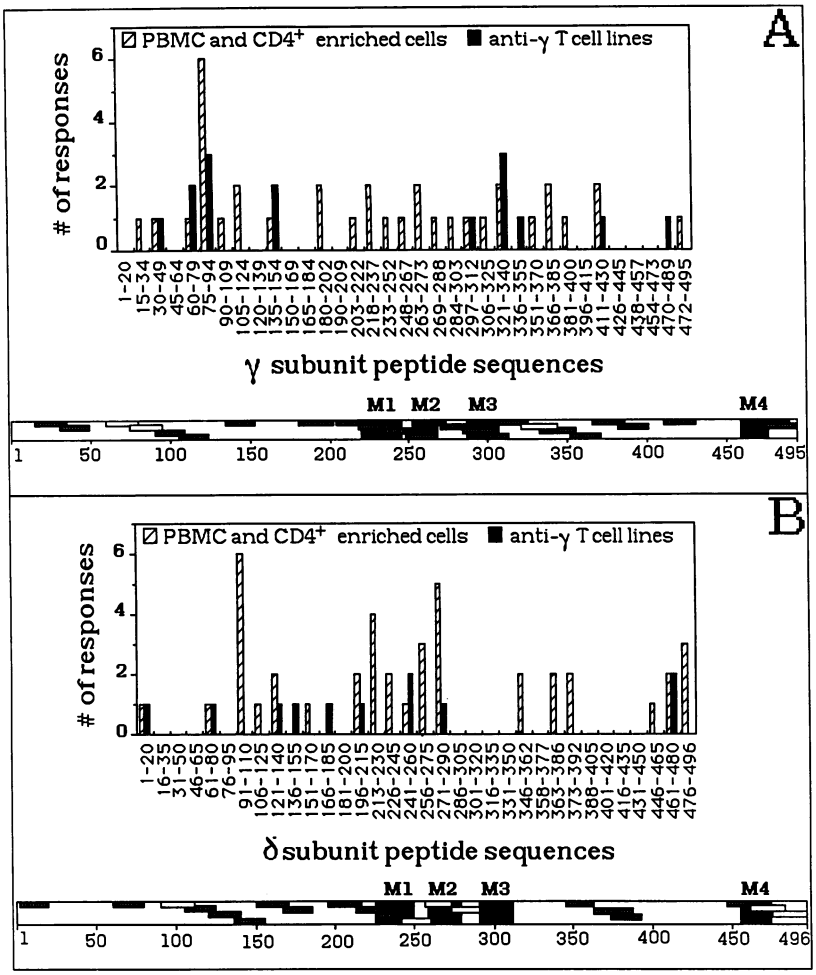


Figure 2. Frequency of recognition of peptide sequences on the γ subunit (*A*) and on the δ subunit (*B*) by the eight responder MG patients of this study (*hatched columns*) and by anti-AChR T cell lines propagated from different MG patients and specific for the γ and δ subunits (*black columns*). At the bottom of each panel, a schematic representation of the location of the sequence regions forming T epitopes on the γ and δ subunit sequences is given. The epitopes forming sequence regions are indicated as gray segments, while white segments indicate immunodominant peptides recognized by patients of different class II haplotype. The four putative transmembrane regions (M1–M4) are indicated in black. The sequence segment aminoterminal to the M1 region (residues 1 to ~ 220) is believed to form an extracellular domain, and the segment between M3 and M4 a cytoplasmic domain. The transmembrane topology of the carboxyl terminal sequence segment following M4 is still under debate but is probably extracellular (reviewed in references 19 and 20; see also reference 82).

nized by patients of different DR haplotype. Because these immunodominant sequences are 20 or more residues long, they may contain nested epitopes, recognized in association with different DR molecules (63–66). Recognition of the same sequence by patients of different DR haplotype may be due to the promiscuous binding of peptides to the human DR molecules (67). The large number of $CD4^+$ epitopes recognized by MG patients on the AChR molecule makes it unlikely that epitope-specific immunosuppressive therapies will be easily and expeditiously developed for MG.

The anti-AChR antibody response in MG is also polyclonal (1–4), and even antibodies directed to the same AChR epitope have different idiotypes (68, 69). However, it is likely that only antibodies against particular AChR epitopes, which should include the MIR (70, 71), cause AChR destruction and myasthenic symptoms, as suggested by the lack of correlation between the antibody titer and the severity of MG (1–4, 72, 73) and by experiments that tested the ability of different anti-AChR (74). It is possible that among the many anti-AChR Th clones present in MG patients, only those directed against particular epitopes are involved in the development of MG symptoms, due to the preferential pairing with B cells secreting antibodies of high pathogenetic potential. Further investigations in mice with genetically determined severe combined immunodeficiency (75–77), xenografted with different combinations of PBMC from MG patients and AChR $CD4^+$ cells of the defined epitope specificity, may resolve this issue.

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