Epitopes on the β Subunit of Human Muscle Acetylcholine Receptor Recognized by CD4+ Cells of Myasthenia Gravis Patients and Healthy Subjects

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Abstract

We investigated the sequence regions of the human muscle acetylcholine receptor (AChR) β subunit forming epitopes recognized by T helper cells in myasthenia gravis (MG), using overlapping synthetic peptides, 20 residues long, which screened the sequence of the AChR β subunit. Since CD4+ lymphocytes from MG patients' blood did not respond to the peptides, we attempted propagation of β subunit–specific T lines from six MG patients and seven healthy controls by cycles of stimulation of blood lymphocytes with the pooled peptides corresponding to the β subunit sequence. CD4+ T lines were obtained from four patients and three controls. They secreted IL-2, not IL-4, suggesting that they comprised T helper type 1 cells.

The T lines from MG patients could be propagated for several months. Three lines were tested with purified bovine muscle AChR and cross-reacted well with this antigen. All T lines were tested with the individual synthetic peptides present in the pool corresponding to the β subunit sequence. Several β subunit peptide sequences were recognized. Each line had an individual pattern of peptides recognition, but three sequence regions (peptides β181–200, β271–290, and the overlapping peptides β316–335 and β331–350) were recognized by most MG lines.

The β subunit–specific T lines from controls could be propagated for < 5 wk. Each line recognized several peptides, which frequently included the immunodominant regions listed above. (*J. Clin. Invest.* 1994. 93:1020–1028.) Key words: synthetic peptides • T cell lines • CD4+ subsets

Introduction

In myasthenia gravis (MG)[1] an autoimmune response against the muscle nicotinic acetylcholine receptor (AChR) occurs (for reviews see references 1–5), with synthesis of AChR antibodies and sensitization of AChR-specific T helper (Th, CD4+) cells. The AChR of mammalian muscle is a complex transmembrane protein, formed by four homologous subunits, α, β, ε (γ in embryonic muscle), and δ (for review see reference 6).

Since Th cells are necessary for synthesis of high affinity anti-AChR IgG antibodies (7) and possibly for maintenance of B cell tolerance by mechanisms of clonal anergy (8), it is important to define and catalog the epitope repertoire of the autoimmune anti-AChR Th cells in MG patients and of potentially autoreactive Th cells able to recognize AChR sequences in healthy subjects. Several sequence regions forming DR-restricted epitopes recognized by AChR Th cells in MG patients have been identified on the α, γ, and δ AChR subunits (for review see reference 9). Some such regions, 20 residues long, are recognized by most MG patients, irrespective of their MHC class II haplotype (10–12).

In this study we investigated whether the AChR β subunit is involved in the sensitization of Th cells and we sought identification of sequence regions of the β subunit forming epitopes recognized by CD4+ cells of MG patients. Since CD4+ cells able to recognize autologous antigens, including the AChR, have been described in healthy subjects (e.g., see references 11–16), we also investigated the existence of anti-AChR β subunit CD4+ cells in healthy controls and their epitope specificity. We used a panel of 32 synthetic peptides, 20 residues long and overlapping each other by 5 residues, corresponding to the complete sequence of the human AChR β subunit (17). We first used these peptides to test the response of unselected CD4+-enriched, CD8+-depleted blood cells. No response could be detected in our patients, who had mild or moderate symptoms. This agrees with previous studies which indicated that only unselected blood cells from severely affected MG patients showed a measurable in vitro response to synthetic AChR antigens (13, 18). To obtain a CD4+ population enriched in β subunit–specific cells, we attempted propagation of T cell lines by cycles of stimulation with the pooled peptides corresponding to the β subunit sequence. Anti-β subunit CD4+ T lines were obtained from four of six patients and from three of seven controls. The sequence regions of the β subunit forming epitopes recognized by the T lines were identified by challenging the lines in microproliferation assays with the individual synthetic peptides.

Methods

Patients and controls. We used 12 MG patients, all suffering from mild or moderate generalized symptoms. In all but one patient, we tested the response of unselected CD8+-depleted, CD4+-enriched blood mononuclear cells to pools of synthetic peptides corresponding to the complete sequence of the human AChR α, β, γ, and δ subunits (17, 19–21) and to individual peptides screening the human β subunit sequence, as described below.

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1. *Abbreviations used in this paper:* AChR, muscle nicotonic acetylcholine receptor; α, β, γ, or δ pool, a pool of synthetic peptides corresponding to the complete sequence of human AChR α, β, γ, or δ subunit; APC, antigen-presenting cells; BAChR, bovine muscle AChR; MG, myasthenia gravis; TAChR, *Torpedo californica* AChR; TCM, tissue culture medium; Th, T helper.

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We attempted propagation of β subunit–specific T cell lines from six patients, whose salient characteristics, HLA-DR haplotype, and serum anti-AChR antibody titer (measured as described in reference 22) at the time when the lines were started are reported in Table I. All patients had undergone thymectomy: patients 3 and 5 had different degrees of hyperplastic thymic abnormalities, which are present in as many as 80% of MG patients (1–5, 9); and patients 2 and 6 had a thymoma. The other two patients (1 and 4) had a seemingly normal thymus. Three patients had been donors of long-term $CD4^+$ T cell lines specific for the human AChR α, γ, and/or δ subunits (10–12), as summarized in Table I.

Propagation of Th cell lines was attempted from seven healthy controls (Table I).

Peptide synthesis and characterization. 32 peptides, 20 residues long and corresponding to the complete sequence of the human AChR β subunit (17), were synthesized (23). The peptides overlapped each other by five residues to minimize the risk of missing epitopes split between peptides and corresponded to the following segments of the β subunit: 1–20, 16–35, 31–50, 46–65, 61–80, 76–95, 91–110, 106–125, 121–140, 136–155, 151–170, 166–185, 181–200, 196–215, 211–230, 226–245, 241–260, 256–275, 271–290, 286–305, 301–320, 316–335, 331–350, 346–365, 361–380, 376–395, 391–410, 406–425, 421–440, 436–455, 451–470, and 466–478. They are indicated by codes, which include the symbol β and two numbers, indicating the position of the first and last peptide residues on the β subunit sequence. The composition of the peptides, determined by phenylthiocarbamoyl derivatization of the amino acids released by acid hydrolysis (24), yielded a satisfactory correspondence between experimental and expected values for all peptides. Several peptides, including the epitope peptides β16–35, β76–95, β376–395, and β181–200, had a low but acceptable yield of basic residues, which may be because of poor coupling during the synthesis or poor deprotection during the cleavage, with consequent apparent low yield of these residues. Table II reports the amino acid composition of the 12 peptides recognized by the $CD4^+$ lines.

Purification of AChR from Torpedo electroplax and bovine muscle. Native, membrane-bound *Torpedo californica* AChR (TAChR) was prepared from electric tissue and was characterized as we described previously (10). The specific activity of TAChR preparations (expressed as nanomoles of ^{125}I–α-bungarotoxin binding sites per milligram of protein) was 4–7 nmol/mg (maximum theoretical activity of pure AChR: 7.2 nmol/mg). Bovine muscle AChR (BAChR) was purified from 8–12-in-long bovine fetuses (25).

$CD8^+$ T cell depletion of PBMC. For the four patients from which β lines could be successfully propagated, for patient 5 from which propagation of a β subunit–specific line was unsuccessfully attempted, and for six other patients not studied otherwise, we tested the response to the β subunit peptides of $CD8^+$-depleted, $CD4^+$-enriched PBMC. $CD8^+$ T cell depletion was done using mouse anti-$CD8^+$ antibody (OKT8; Ortho Pharmaceutical, Raritan, NJ) and paramagnetic beads coated with goat anti–mouse Ig antibody (Advanced Magnetics Inc., Cambridge, MA), as we described previously (18). The $CD8^+$-depleted, $CD4^+$-enriched cells obtained (referred to as $CD4^+$-enriched PBMC) were consistently 45–55% of the starting PBMC. The cellular composition of the $CD4^+$-enriched PBMC was determined by FACS® analysis in four pilot experiments and was ($n = 4$): T cells ($CD3^+$), 61.4±15%; $CD4^+$ cells, 55±14.3%; $CD8^+$ cells, 0.5±0.1%.

Propagation of Th cell lines specific for the AChR β subunit. The pool of synthetic peptides corresponding to the sequence of the β subunit of the human AChR (β pool) was used to propagate AChR-specific T cell lines. PBMC ($2–4 \times 10^7$) were suspended ($1–2 \times 10^6$ cells/ml) in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated human AB serum (Sigma Immunochemicals, St. Louis, MO), 2 mM L-glutamine, 100 U/ml penicillin, and 50 μg/ml streptomycin (tissue culture medium, TCM), containing β pool at a concentration of 1 μg/ml of each peptide, and were cultivated in T25 flasks (Costar Corp., Cambridge, MA) for 1 wk. The reactive lymphoblasts were isolated on Percoll gradients (10), were further expanded in TCM containing T cell growth factor (Lymphocult; Biotest Diagnostic Inc., Dreieich, Germany) at a final concentration of IL-2 of 10 U/ml, and were enriched in β subunit–specific cells by weekly stimulations with the same amount of β pool plus irradiated (4,000 rads: 1 rad = 0.01 Gy) autologous (for the control lines) or HLA-DR–matched (for the Th cell lines from MG patients) PBMC as antigen-presenting cells (APC). Autologous or DR-matched PBMC are equally good APC for propagation of anti-AChR Th cell lines, which are DR restricted (26, and Moiola, L., M. P. Protti, and B. M. Conti-Tronconi, unpublished observation). The T cell lines obtained were tested weekly for their ability to respond to the β pool and to PHA. The enrichment in β pool reactivity was considered satisfactory when the response to the β pool was comparable with or higher than that to PHA. This occurred after 3–4 wk of culture.

Flow cytometry. The phenotype of the T cell lines and of the $CD4^+$-enriched PBMC was determined by FACS® analysis in a FACStar® cell sorter (Becton Dickinson and Co., Mountain View, CA) by using phy-

Table I. Patients and Controls Used in This Study

	Age, sex	Symptoms*	Antibody titer	Thymus pathology‡	Treatment§	HLA-DR haplotype¶	Successful propagation of AChR subunit-specific Th lines			
							anti-α	anti-β	anti-γ	anti-δ
			nM							
Patients										
1	36, F	Mild generalized	0.06	Normal	Pred	DR1, DRw14(6)	No	Yes	Yes	Yes
2	59, M	Mild generalized	4.22	Thymoma	AChE/Pred/AZA	DRw17(3), DR7	Yes	Yes	No	Yes
3	47, F	Mild generalized	1.78	Hyperplasia	AChE/Pred/CYA	DRw15(2), DR4	Yes	Yes	Yes	No
4	25, F	Mild/moderate generalized	4.51	Normal	AChE/Pred	DR9, DRw8	NA**	Yes	NA**	NA**
5	25, F	Moderate generalized	8.41	Hyperplasia	AChE/Pred/Plex/IVIG	DRw15(2), DR4	NA**	No	NA**	NA**
6	61, F	Moderate generalized	1.76	Thymoma	AChE/AZA/Plex	DR1, DRw15(2)	NA**	No	NA**	NA**
Controls										
1	27, F					Drw11(5), DRw17(3)				
2	27, M					DRw15(2), DR4				
3	40, F					DRw15(2), DR9				
4	36, M					DRw11(5), DRw17(3)				
5	30, M					DR1, DR4				
6	44, F					DRw11(5), DRw15(2)				
7	30, M					DRw12(5), DRw15(2)				

* Classified as described in reference 51. ‡ All patients had undergone thymectomy. § AZA, azathioprine; Plex, plasma exchange; IVIG, intravenous immunoglobulins; CYA, cyclosporine; AChE, anticholinesterase drugs. ¶ Determined by RFLP (52). ** NA, not attempted.

coerythrin-conjugated Leu 2 (anti-CD8), Leu 3 (anti-CD4), and Leu 4 (anti-CD3) antibodies (Becton Dickinson and Co.). Dilutions, washings, and incubations were done in PBS at 4°C.

Microproliferation assay. The T-line cells (blasts) were diluted to 2×10^5/ml in TCM. HLA-DR–matched, irradiated (4,000 rad) PBMC, to be used as APC, were diluted to 2×10^6 in TCM. The cells were plated in triplicate in 96 round-bottom-well plates (100 μl blasts plus 100 μl APC) and were stimulated with one of the following antigens: pools of overlapping synthetic peptides corresponding to the complete sequence of the human muscle AChR α, β, γ, and δ subunit (α pool, β pool, γ pool, and δ pool; 0.05, 0.1, 0.5, 1, and 5 μg of each peptide/ml); each of the 32 individual synthetic peptides forming the β pool (10 μg/ml); PHA (Wellcome Reagent Ltd., London, UK; 10 μg/ml); T cell growth factor (Lymphocult; Biotest Diagnostic Inc.: final concentration of IL-2, 10 U/ml); TAChR (10, 20, and 40 μg/ml); and in some experiments BAChR (10, 20, and 40 μg/ml). Triplicate wells with blasts alone and three wells with APC alone were seeded as controls. The basal growth rate (blanks) was determined from triplicate wells with blasts plus APC that did not receive any stimulus, and/or triplicate wells with blasts plus APC and a 19-residue synthetic peptide unrelated to the human β subunit (E 73, residues 1–19 of the major intrinsic protein of bovine lens [27]; 10 μg/ml). After 24 h, the cells were labeled with [^{3}H]thymidine (1 μCi per well; sp act 6.7 Ci/mmol; Amersham Corp., Arlington Heights, IL) for 16–18 h and were collected with a multiple harvester (Titertek; Skatron, Inc., Sterling, VA). Incorporation of [^{3}H]thymidine was measured in a liquid scintillation counter.

Similar microproliferation assays were carried out with CD4$^+$-enriched PBMC, using 1×10^5 cells/well and labeling with [^{3}H]-thymidine after 96 h (18).

Assay of IL-2 and IL-4 secretion. Secretion of IL-2 and IL-4 by the Th cells was determined with a commercial ELISA kit (Quantikine human IL-2 and IL-4; Immunoassay Research and Diagnostic Systems, Minneapolis, MN), using supernatant obtained at the end of the first period of stimulation of PBMC with the β pool and/or supernatant obtained after several cycles of stimulation of the Th lines with β pool.

Results

No response to β subunit epitopes can be detected using unselected CD4$^+$-enriched PBMC. Testing of unselected, CD4$^+$-enriched PBMC did not yield measurable responses to the β pool, or to individual β subunit peptides, or to the α, γ, and δ peptide pools. Since the patients we studied suffered from mild or moderate symptoms, the negative results agree with those we obtained previously in studies investigating the Th response to the α, γ, and δ subunits, which indicated that a response to AChR epitopes of unselected blood CD4$^+$ cells could be detected only in patients suffering from severe symptoms (13, 18, 28).

Propagation of β subunit–specific lines from MG patients. Propagation of β subunit–specific Th lines was successful in four out of six patients (Patients 1–4, see Table I). The lines were considered sufficiently enriched in specific Th cells when their response to the β pool in microproliferation assay was comparable with that to PHA. This occurred after approximately three to four cycles of stimulation with β pool. The Th lines could be propagated for several months, although the pattern of peptide recognition sometimes became more simplified after a longer period of propagation, probably as a result of clonal loss. The phenotype of the Th cell lines is reported in Table III: they were predominantly or exclusively CD3$^+$, CD4$^+$, and CD8$^-$.

Table II. Amino Acid Composition of Synthetic Peptides Forming CD4$^+$ Epitopes on the Human β Subunit Sequence

	Hβ16–35		Hβ31–50		Hβ76–95		Hβ181–200		Hβ271–290		Hβ316–335		Hβ331–350		Hβ361–380		Hβ376–395		Hβ391–410		Hβ406–425		Hβ436–455	
	Ex	Fd	Ex	Fd	Ex	Fd	Ex	Fd	Ex	Fd	Ex	Fd	Ex	Fd	Ex	Fd	Ex	Fd	Ex	Fd	Ex	Fd	Ex	Fd
D, N	2	2.3	2	2.4	4	4.6	3	4.1	1	0.7	0	0	1	1.1	2	2.1	2	2.2	3	2.9	0	0	2	2.1
T	0	0	0	0	1	0.9	0	0	1	1.1	1	0.9	0	0	1	1	0	0	0	0	0	0	1	1
S	3	3.2	2	1.6	2	1.7	1	1	2	1.4	0	0	0	0	1	1	1	0.9	0	0	3	2.8	0	0
E, Q	1	1.1	4	3.8	1	0.8	4	3.7	1	0.9	2	2.1	2	2.1	1	0.8	2	2.1	1	0.8	4	4.3	1	1.1
P	1	1	0	0	1	1.1	3	3.7	2	2.2	2	1.9	5	5.5	3	3.2	4	4.1	3	2.9	1	1	0	0
G	2	2.8	1	0.6	0	0	2	2.1	0	0	0	0	0	0	2	2.2	0	0	1	1.1	0	0	0	0
A	1	1.2	1	1.3	1	1.2	0	0	1	1	0	0	0	0	0	0	1	1.2	2	2.3	1	1.3	2	2.4
V	5	6.3	2	2	3	2.6	0	0	2	1.9	1	0.9	0	0	0	0	0	0	1	1	2	1.7	3	2.7
M	0	0	1	0.9	0	0	0	0	1	1	1	0.6	1	0.9	0	0	0	0	0	0	0	0	1	1.1
I	0	0	4	4.3	1	0.9	2	2	3	2.7	2	1.9	0	0	1	1	0	0	1	0.9	2	2.1	2	1.8
L	1	1.5	0	0	4	4.3	1	1.2	3	3.5	4	4.6	5	4.9	1	1.2	3	3.5	4	4.1	4	4.5	2	2.2
Y	0	0	0	0	0	0	0	0	1	1	1	1.1	1	0.9	1	0.9	0	0	0	0	1	0.7	0	0
F	0	0	0	0	0	0	0	0	0	0	1	0.8	0	0	3	3.3	3	3.2	1	0.9	0	0	4	4.2
H	0	0	1	1.2	0	0	1	0.9	0	0	2	2	0	0	0	0	0	0	0	0	0	0	0	0
K	0	0	0	0	0	0	1	0.9	2	2.3	1	1.1	2	2.1	2	1.4	1	0.6	0	0	0	0	0	0
R	4	2.1	0	0	1	0.6	1	0.6	0	0	1	1.2	3	3.2	2	1.9	3	2.6	3	3.3	2	1.8	0	0

*Table III. Phenotype of the β Subunit-specific T Cell Lines**

Line	CD3+	CD4+	CD8+
	%	%	%
Patients			
1	96.8	89.4	12.2
2	97.7	97.0	1.8
3	84.9	82.3	0.8
4	96	92	5.4
Controls			
1	96.8	88.5	6.3
2	83.8	41.9	44.4
3	98.5	95.6	5.7

* Determined by FACS® as described in Methods, after 4–6 (for MG lines) and 3 (for the control lines) wk of propagation.

The lines responded to the β pool vigorously, and in a dose-dependent manner (Fig. 1). Doses of β pool of 0.1–0.5 μg of each peptide/ml elicited significant responses in all lines, and the responses were close to maximum at 1 μg of each peptide/ml (Fig. 1). The specificity of the lines was verified by testing their response to pools of similar overlapping synthetic peptides corresponding to the complete sequences of the α, γ, and δ subunits of human muscle AChR, to TAChR, and to BAChR. BAChR is a good substitute for the exceedingly scarce human muscle AChR because it can be purified in sufficient amounts (25) and is highly homologous to human AChR (for review see reference 6). The bovine and human β subunits are 92% identical (for review see reference 6). All lines responded specifically to the β pool and cross-reacted minimally or not at all with the other AChR subunit peptide pools (Fig. 2). The lines did not cross-react with TAChR (data not shown). This might be expected from the limited sequence identity of *Torpedo* and human β subunits (~ 55%, see references 17 and 6).

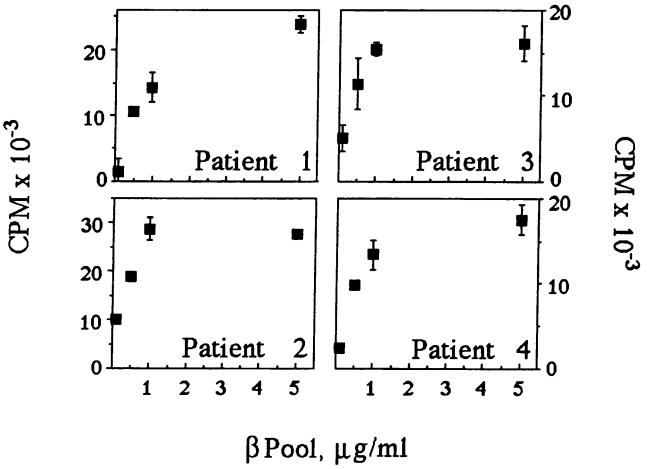

Figure 1. Dose dependence of the response to the β pool of the four CD4+ lines obtained from MG patients, measured by in vitro microproliferation assays. The data are means of triplicate determinations ±SD. The basal incorporation of [3H]thymidine, in the absence of any antigen stimulus, has been subtracted and it was: line 1: 6,280±620; line 2: 2,410±1,160; line 3: 8,720±370; line 4: 470±20. See text for experimental details.

Because of the scarcity of purified BAChR, only three of the four β lines could be tested with this antigen (lines 1, 3, and 4). They all responded to BAChR, although to different extents (Fig. 2, *insets*). The response of lines 1 and 3 to BAChR was strong and was even larger than that induced by PHA, in spite of the high basal rate of cell proliferation of line 3, which suggests that the T blasts had not yet reverted to the resting state, susceptible to restimulation with the antigen when the test was carried out. The response of line 4 to BAChR was significant ($P < 0.001$) but small, perhaps because of incomplete reversion of the blasts to the resting state (Fig. 2, *inset*), as suggested by the high basal rate of proliferation.

Propagation of Th cell lines with β pool from controls. T cell lines specific for the β subunit could be propagated by stimulation of PBMC with β pool from three of the seven controls. The phenotype of the three control lines determined by FACS® analysis is reported in Table III. Lines 1 and 3 were predominantly CD3+, CD4+, and CD8−, while line 2 comprised approximately equal numbers of CD4+ and CD8+ cells. Although all control T lines responded vigorously and specifically to the β pool (Fig. 3), they could be propagated for a short time only; after 4–5 wk of propagation, the lines stopped proliferating in response to the β pool and died.

The β subunit-specific lines comprised only Th1 cells. The CD4+ subsets present in the β subunit-specific lines from MG patients and controls were assessed by determining their ability to secrete IL-2 and IL-4 (products of Th1 and Th2 cells, respectively) (29, 30). All lines secreted IL-2 only, indicating that they comprised only or predominantly Th1 cells (Table IV).

Sequence segments of the β subunit recognized by MG patients. The segments of the β subunit forming epitopes recognized by the CD4+ T cell lines were identified by challenging the lines with the individual synthetic peptides present in the β pool. To minimize loss of epitope recognition resulting from biased clonal selection during propagation of the lines, the lines were tested for reactivity to individual synthetic peptides as soon as a satisfactory enrichment in reactivity to the β pool was obtained, i.e., when the response of the lines to the β pool in microproliferation assay was comparable with that to PHA (Fig. 2), after 3–4 wk of culture. The consistency of the recognition was verified by repeating the test every 1–2 wk during the first 2 mo of propagation of the lines.

Fig. 4 *A* illustrates the pattern of peptides recognition of the β subunit-specific line 4 during the first 2 mo of propagation. During the early stages of propagation of the line (2 wk), the responses to epitope-forming sequences (sequences β16–35, β181–200, β271–290, and the overlapping peptides β316–335 and β331–350), although significant, are barely discernible above the fluctuating baseline. After 3 wk, the baseline level of proliferation to peptides which do not form epitopes was still high but was more uniform. Some sequences elicited vigorous responses (peptide β181–200, and the overlapping peptides β316–335 and β331–350), while others induced weak but significant responses (e.g., β436–455). At difference with the other lines, which recognized the largest number of β subunit sequences when the test was carried out after 3–4 wk of propagation, line 4 after 3 wk of propagation did not respond to the sequence regions β16–35 and β271–290, which elicited small but significant responses in the assays carried out after 2 and 7 wk of propagation (see above and below). After 7 wk, the basal proliferative response in the absence of AChR sequences or in presence of β sequences which do not form epitopes for this

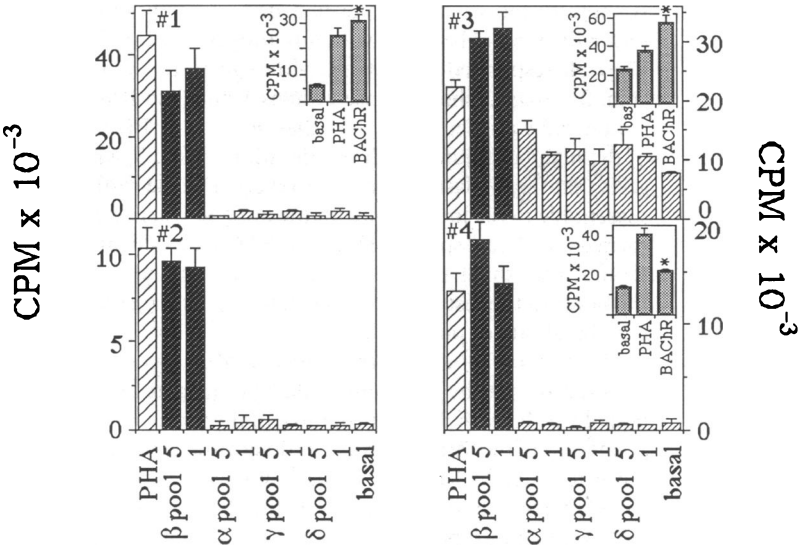

Figure 2. The CD4+ lines propagated from MG patients specifically recognized the β pool and BAChR. The lines were challenged in microproliferation assays with different concentration of β, α, γ, and δ pools (1 and 5 μg of each peptide/ml, as indicated along the abscissa). All lines recognized the β pool only, and the response to this antigen was comparable with that elicited by PHA. The insets depict the response of the lines to purified BAChR (20–24 μg/ml): the response to BAChR of lines 1 and 3 was vigorous, that of line 4 was weak but significant. The basal [^{3}H]-thymidine incorporation of unstimulated cells was not subtracted and is reported (*basal*). The stars indicate responses significantly higher ($P < 0.001$, as determined by unpaired, one-tailed Student's t test) than the unstimulated proliferative rate of blasts in the presence of APC only, and in the presence of APC plus 10 μg of the unrelated peptide E 73. See text for experimental details.

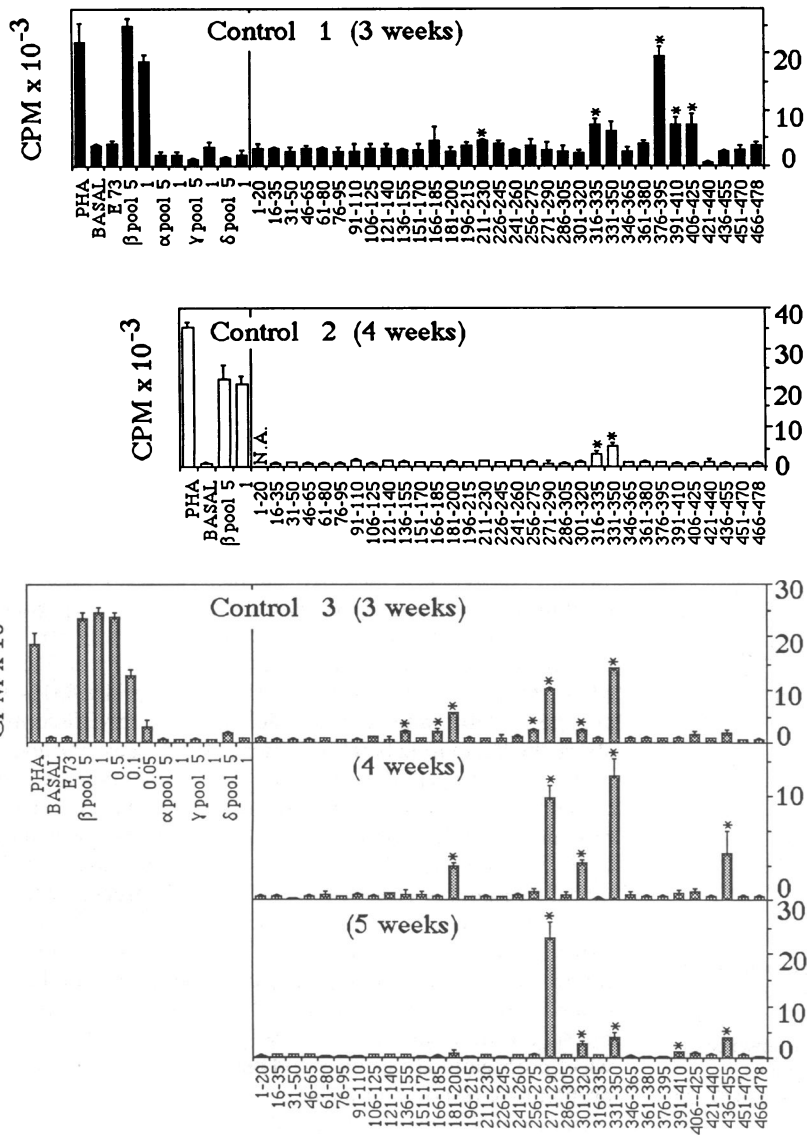

Figure 3. CD4+ lines propagated from healthy controls specifically recognized β pool and individual β subunit peptides. The lines were tested after 3–5 wk of propagation, as indicated in the figure. In the left part of the panel, we report the response to different concentration of β pool (micrograms of individual peptides per milliliter, as indicated along the abscissa) and, for lines 1 and 3, to different concentrations of α, γ, and δ pools. BASAL indicates [^{3}H]thymidine incorporation of unstimulated cells which has not been subtracted from the data. E 73 (control lines 1 and 3) represents [^{3}H]thymidine incorporation of T blasts plus APC plus the control peptide E 73. All lines specifically and vigorously recognized the β pool to an extent comparable with the response to PHA. The right side of the figure depicts the response of the lines to the individual synthetic sequence regions of the β subunit (10 μg/ml), as indicated along the abscissa. Only control line 3 could be tested more than once. Several peptides were recognized by each line. The values are means of triplicate determinations ±SD. The stars indicate responses to the individual peptide significantly different ($P < 0.001$, as determined by unpaired, one-tailed Student's t test) from the baseline [^{3}H]-thymidine incorporation in the absence of any peptide and in the presence of at least 10 β subunit peptides which were not recognized by the T lines. See text for experimental details.

*Table IV. Ability of β Subunit-specific Lines to Secrete IL-2 and IL-4**

Line	IL-2	IL-4
	pg/ml	*pg/ml*
Patients		
1	33.6	0
2	32	0
3	96.3	0
4	163	0
Controls		
1	43.6	0
2	26.1	0
3	34	0

* The interleukin content in the supernatant of cell culture after stimulation with β pool was determined as described in Methods.

line was low and uniform. Four sequence regions elicited significant responses above the background (listed in decreasing order of blasts response): the overlapping peptides β316–335 and β331–350, and peptides β181–200, β271–290, and β16–35.

The response to the β subunit sequences of the other three lines was most clear and complex after 3–4 wk of propagation (Fig. 4 *B*). Line 1 strongly recognized peptides β181–200, β316–335, and the overlapping peptides comprising the sequence region β361–410. Peptide β31–50 elicited a small but significant response. In two other experiments, not reported in Fig. 4 and carried out after 3 and 5 wk of propagation, the line recognized weakly but significantly also peptide β271–290 (Fig. 5). Line 2 strongly recognized peptides β181–200, β271–290, and β316–335. Peptides β76–95, β331–350 (which overlaps the strong stimulator β316–335), and β361–380 elicited small but significant responses. Line 3 always strongly recognized peptide β331–350. Peptides β241–260, β406–425, and β436–455 elicited marginal responses at the beginning of the propagation. As the propagation of the line proceeded, its response was limited to peptides β331–350 and β406–425.

Sequence regions of the β subunit recognized by CD4⁺ lines from healthy controls. The three β subunit-specific lines propagated from healthy controls were challenged with the individual peptides screening the β subunit sequence (Fig. 3).

Control lines 1 and 2 grew poorly and could be tested with the individual peptides only once, shortly before the line stopped growing. Control line 1 recognized the sequence β376–395 strongly and the sequences β211–230, β316–335, and the overlapping peptides β391–410 and β406–425 to a low extent. Control line 2 recognized the two overlapping peptides β316–335 and β331–350. Control line 3 had a good growth rate, which allowed repetitive testing after 3, 4, and 5 wk of propagation. The line clearly and consistently recognized several peptides: β181–200, β271–290, β301–320, β331–350, and β436–455.

Discussion

In this paper we report sequence regions of the human muscle AChR β subunit recognized by autoimmune or potentially autoimmune Th cells by extending to this subunit experimental approaches we successfully used for the α, γ, and δ subunits (10–12). We used overlapping synthetic peptides corresponding to the complete sequence of a given AChR subunit to propagate AChR-specific CD4⁺ Th cell lines and to identify the sequence regions forming the epitopes they recognize. β subunit-specific CD4⁺ lines were obtained from four of six MG patients and from three of seven controls.

All lines recognized the β pool specifically and vigorously. The CD4⁺ lines from MG patients are AChR specific and they recognized epitopes produced from processing of the AChR

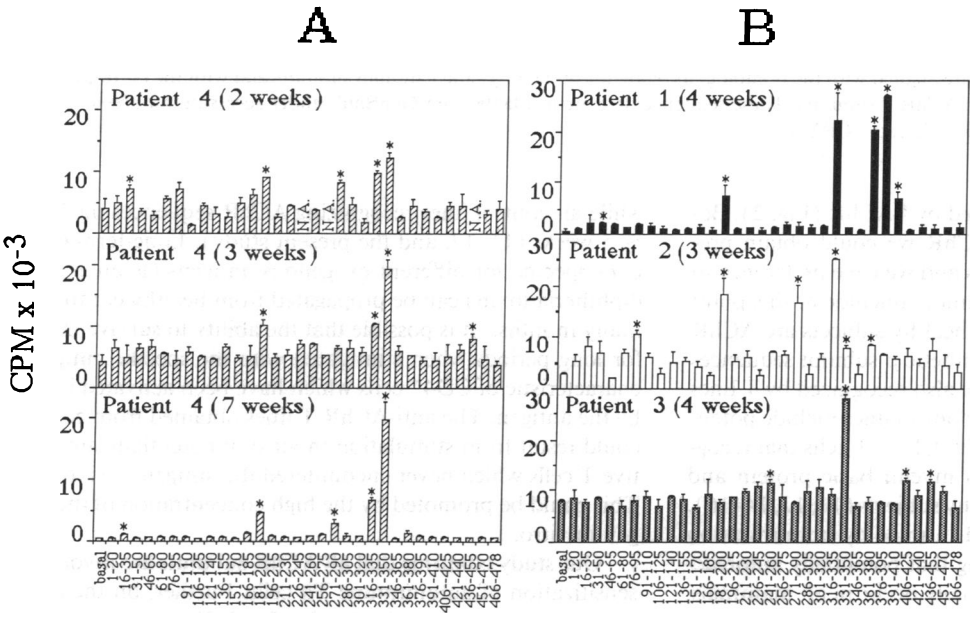

Figure 4. Response of anti-β subunit CD4⁺ T cell lines from MG patients to the individual synthetic peptides screening the β subunit sequence (10 μg/ml), as indicated along the abscissa, measured in 2-d microproliferation assays. The values are means of triplicate determinations ±SD. Panel A illustrates the pattern of peptide recognition of line 4 during the first 2 mo of propagation. In the first two experiments (after 2 and 3 wk of propagation) the baseline proliferation in the presence of peptides which do not form epitopes was high, while in the test carried out after 7 wk was low and uniform. Panel B reports the response to the β subunit sequences of the other three lines (1, 2, and 3) after 3 or 4 wk of propagation, when the response was most clear and complex. Basal represents [³H]-thymidine incorporation of unstimulated cells, which has not been subtracted from the data. The stars indicate the responses significantly different ($P < 0.001$, as determined by unpaired, one-tailed Student's t test) than the baseline incorporation of [³H]thymidine in the absence of any peptide and in the presence of at least 10 β subunit peptides which were not recognized by the T lines. See text for experimental details.

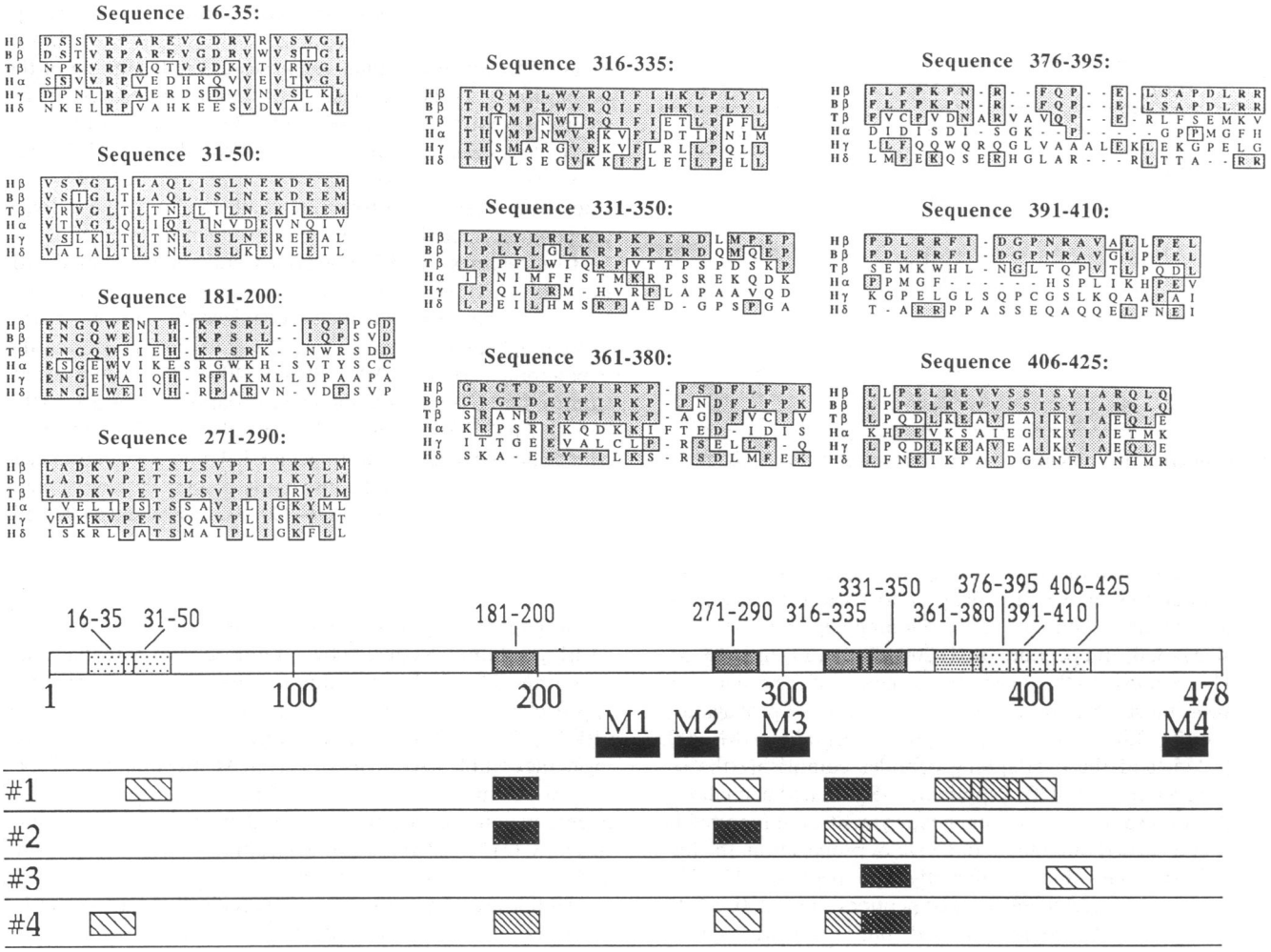

Figure 5. Summary of the sequence segments recognized by the four T cell lines from MG patients. (*Bottom*) The peptides that elicited a strong response are indicated for each line as dense hatched segments, and the peptides that elicited lower responses are indicated as increasingly lighter hatched segments. (*Middle*) The sequence segments forming epitopes are aligned along a diagram of the β subunit where the epitope regions are indicated as segments of increasing darkness, reflecting the frequency with which they were recognized by the MG lines. The four sequence regions of the β subunit proposed to form transmembrane α helices, M1–M4, are also indicated (*black segments*). (*Top*) The sequence segments of the β subunit containing Th epitopes are aligned with the homologous segments of the α, γ, and δ human subunits and with the corresponding segments of bovine muscle and *Torpedo* AChR β subunits. These sequence data are available from GenBank under accession numbers A27591, S04607, A23261, X55019 (mRNA), S07227, and ACRYB1.

molecule because they are stimulated by BAChR (Fig. 2). Because the limited amounts of BAChR we could obtain precluded extensive testing of the lines and we gave preference to the lines from MG patients, we cannot conclude at this point that the β pool–specific lines from healthy subjects are AChR specific. However, since several synthetic β subunit sequences recognized by the control lines were also recognized by T lines of MG patients, it is likely that the control lines include potentially autoreactive anti-AChR T cells. $CD4^+$ T cells that recognize defined autoantigens such as myelin basic protein and AChR have been described in healthy subjects (11, 12, 14–16). Experimental MG can be induced in mice by immunization with rodent AChR (31), suggesting that potentially autoreactive anti-AChR Th cells may normally occur in all mammals.

Do the anti-AChR $CD4^+$ cells of MG patients and healthy subjects differ in their recognition of the antigen and functional properties? Anti-AChR $CD4^+$ lines of MG patients could be propagated for several months (10–12, and the present study), while all control lines recognizing AChR sequences died after $\sim$ 5 weeks (11, 12, and the present study). Long-term $CD4^+$ lines specific for different exogenous antigens (tetanus toxin, diphtheria toxin) can be propagated from healthy controls for many months.[2] It is possible that the ability to survive in vitro for long periods when stimulated with the specific antigen is characteristic of $CD4^+$ cells which have been activated in vivo by the antigen. The anti-AChR T lines obtained from controls could result from stimulation in vitro of potentially autoreactive T cells which never encountered the antigen in vivo (32). This could be promoted by the high concentration of the antigen in vitro.

Our study confirms that all AChR subunits are involved in sensitization of autoimmune Th cells. Further, on the β subunit, as on the α, γ, and δ subunits (9–13, 28), several sequence

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regions are involved in sensitization of $CD4^+$ cells. This strongly suggests that, when generalized MG symptoms have developed, the autoimmune response is directed against all or most of the AChR molecules, but it remains possible that MG might be triggered by cross-reactivity between a microbial epitope and a Th epitope on the AChR (33, 34) or that the anti-AChR response might be initially focused on the embryonic γ subunit, which is expressed in thymus (35) and extraocular muscles (36), two tissues selectively and sometimes uniquely involved in MG. The β subunit epitopes identified here further increase the large catalog of AChR epitopes previously identified on the α, γ, and δ subunits (9–13, 28), thus underscoring the polyclonality of the autoimmune $CD4^+$ response in MG.

Two of the four lines obtained from MG patients (lines 2 and 3) were exclusively $CD3^+$, $CD4^+$, and $CD8^-$, as has been described for T cell lines obtained from MG patients and specific for the α, γ, and δ subunits (10–12), while the other two lines from MG patients (lines 1 and 4) and two control lines (lines 1 and 3), although mostly $CD4^+$, contained a measurable component of $CD8^+$ cells ($\sim$ 5–12%, see Table II). The third control line (line 2) comprised approximately equal numbers of $CD4^+$ and $CD8^+$ cells. Since anti-AChR cytotoxic phenomena are minimal or absent in MG (1–5), it is unlikely that the $CD8^+$ cells propagated here are AChR-specific cytotoxic T cells. $CD8^+$ cells exerting immunosuppressive influences on the anti-AChR $CD4^+$ cells exist in MG (18, 37) and in murine experimental MG (e.g., see 38). The $CD8^+$ cells present in relatively short-term lines (the FACS® analysis reported in Table II was done after 3–5 wk of propagation) might be immunosuppressive $CD8^+$ cells, copropagated with the AChR-specific $CD4^+$ blasts.

The $CD4^+$ lines described here comprised predominantly or exclusively Th1 cells, since they secrete IL-2 and not IL-4. The absence of Th2 cells is not because of the propagation procedure, because the same procedure yielded tetanus toxoid–specific lines from healthy controls which frequently included Th2 cells only.[2] Stable Th1 and Th2 subsets exist in humans (39). Th1 cells can provide help for synthesis of a broad spectrum of Ig classes and subclasses (39), as would be necessary for the anti-AChR response, which includes IgM and IgG of different subclasses (1–5). Th1 cells may have cytolitic activity (30, 39). Given the absence of cytotoxic phenomena in MG (1–5), it is unlikely that the cells described here are anti-AChR cytolitic Th1 cells.

Fig. 5 summarizes the sequence regions of the AChR β subunit forming epitopes recognized by the Th lines from MG patients. The four sequence regions of the β subunit proposed to form transmembrane α helices, M1 to M4 (for review see reference 6), are indicated. The sequence region amino-terminal to M1 is believed to form a large extracellular domain, and the region between M3 and M4 forms a cytoplasmic domain (for review see reference 6). The sequence regions of the β subunit forming T epitopes do not correspond to putative transmembrane segments. Two epitope sequences are part of the putative extracellular domain amino-terminal to M1. One includes the short extracellular sequence segment between M2 and M3, and all the others are clustered in the putative cytoplasmic domain between M3 and M4. Although a relatively large number of β subunit peptides are recognized by the individual T lines, some sequence regions of the β subunit (Fig. 5) form an epitope, or nested epitopes, recognized by most lines, regardless of their HLA-DR haplotype (sequences β181–200, β271–290, and β316–350). Other sequence regions were recognized by two different haplotype lines (sequences β16–50 and β361–380). Sequence regions immunodominant for Th sensitization have been identified on the AChR α, γ, and δ subunits (10, 11, 13, 28). A polyclonal T cell response focused on a relatively small number of immunodominant sequence regions also occurs in normal human Th responses to exogenous antigens (40, 41, and Diethelm, B., R. Raju, and B. M. Conti-Tronconi, manuscript in preparation). This could be related to the promiscuous binding of human DR molecules to different peptides (42, 43). It is possible that sequence regions which form continuous ribbons of residues on the surface of a protein antigen are most easily stripped from the antigen molecule, since that would not require extensive denaturation of the antigen. They would occupy the DR molecules first, outcompeting sequence regions released with slower kinetics.

The anti-AChR antibody response in MG is polyclonal (1–5), and even antibodies directed to the same AChR epitope have different idiotypes (44, 45). However, it is likely that only antibodies against particular epitopes cause AChR destruction and myasthenic symptoms. This is suggested by the low correlation between anti-AChR antibody titer and severity of MG (1–5, 46, 47) and by the differential ability of anti-AChR monoclonal antibodies to cause accelerated destruction of muscle AChR (48). It is possible that among the many anti-AChR Th clones present in MG patients only those directed against particular epitopes and preferentially paired with B cells secreting antibodies of high pathogenic potential are involved in the development of MG symptoms. Further investigations in mice with genetically determined severe combined immunodeficiency (49, 50) that are xenografted with different combinations of PBMC from MG patients and anti-AChR $CD4^+$ cells of the defined epitope specificity may resolve this issue.

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