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Research Article

Prominent T-Cell Responses against the Acetylcholine Receptor ε Subunit in Myasthenia Gravis

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The human acetylcholine receptor (AChR) is well characterized as the target antigen in myasthenia gravis (MG). Pathogenic antibody responses against the AChR alpha-chain have been investigated extensively and are of diagnostic and prognostic value. However, less is known on the pathogenetic relevance of T-cell responses against epitopes of the different AChR chains (alpha, epsilon, gamma). Using an enzyme-linked immunospot (ELISPOT) assay we measured T-cell responses against recombinant fragments and synthetic peptides of the α and the ε subunits of the human AChR in MG patients (n=15) and in healthy donors (HD; n=9). In MG, highest T-cell responses were noted against recombinantly expressed Epsilon 1-221. Among the synthetic peptides Epsilon 201-215 showed the most prominent T-cell response and represented the peptide with the most remarkable difference between MG and HD. Taken together, prominent T-cell responses against the ε subunit of the human AChR indicate an important role in the pathogenesis of MG.

1. Introduction

The human nicotinic acetylcholine receptor (AChR) composed of five subunits ($2\,\alpha, 1\,\beta, 1\,\delta$, and either $1\,\gamma$ or $1\,\varepsilon$ subunit) is well characterized as the target antigen in myasthenia gravis (MG) [1]. The γ subunit of the fetal receptor is replaced by an ε subunit in adult muscle; both subunits share about 53% homology at the amino acid level [2]. Pathogenic antibodies are predominantly directed against the α subunit of the AChR. Both antibody responses as well as B-lymphocyte activity have been investigated extensively in MG and are of great diagnostic and prognostic value.

Immunoglobulin G (IgG) autoantibody production is T helper cell-dependent. Although MG is considered a prototypic paradigm for an antibody-driven autoimmune disorder, the pathogenetic importance of T-helper cells is well appreciated. Several studies have been performed comparing T-cell responses involving the α subunit versus the developmentally regulated ε subunit using recombinant fragments and purified polypeptides of the human AChR [2–8]. The ε subunit is of particular interest as its expression in the adult muscle differs from the fetal γ subunit, a fact that may contribute to the escape of clonal deletion and the development of autoreactive T lymphocytes in MG, especially in the myasthenic thymus [9]. In accordance with this hypothesis, two reports describe that, in comparison to healthy subjects, only MG patients responded to synthetic peptides of the ε subunit by T-cell proliferation [2, 10]. Consistently, in MG patients with thymomas the ε subunit is preferentially expressed [11].

We used an enzyme-linked immunospot (ELISPOT) assay to determine T-cell responses against recombinant fragments and synthetic peptides of the human AChR. In accordance with previous observations we found prominent T-cell responses against the ε subunit while no significant differences were notable against alpha subunit epitopes.

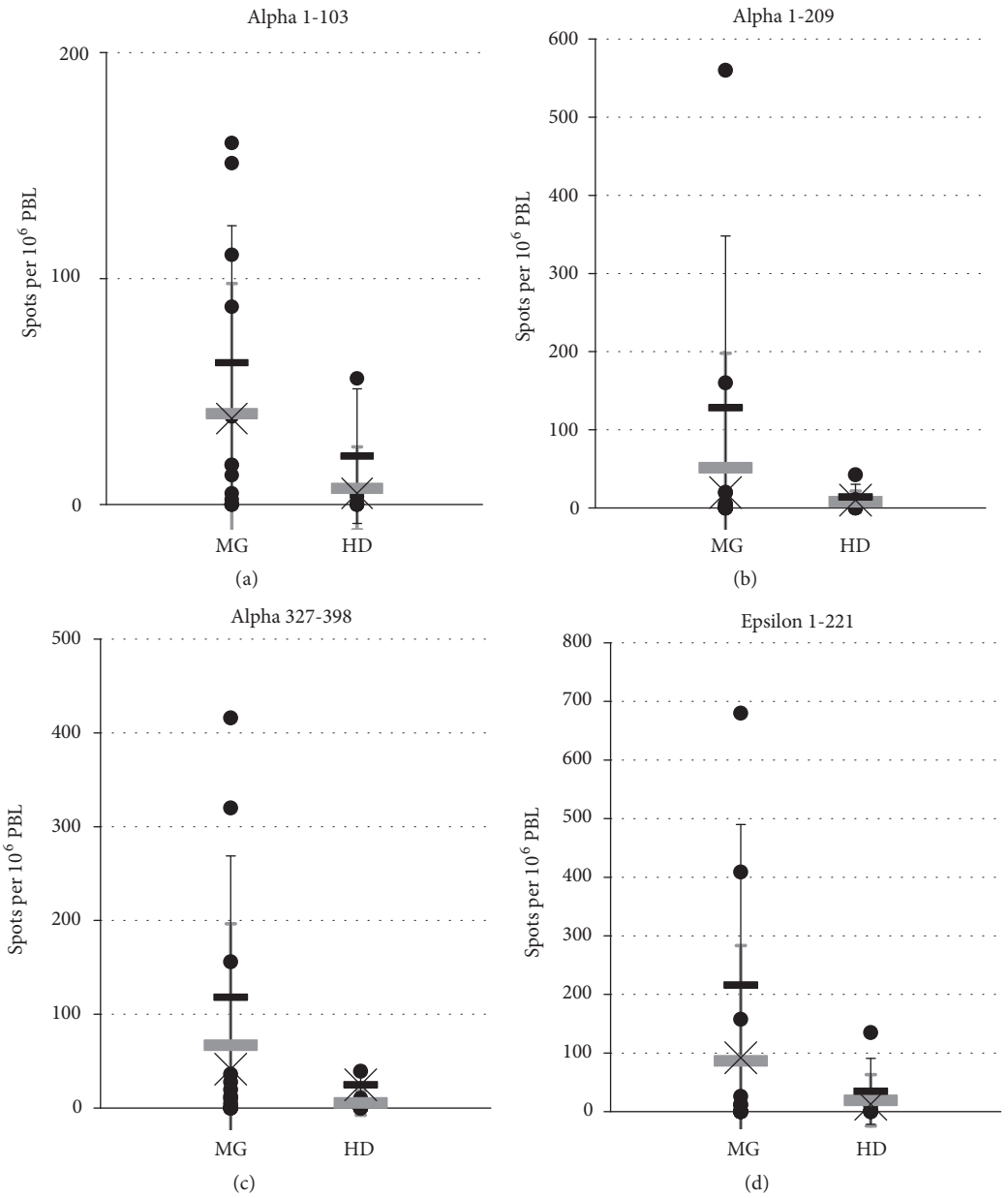

Figure 1: Frequency of IFN-γ-secreting events per 10^6 PBL in single donors. ELISPOT assay was performed as described in the text. Results are assigned as the numbers of IFN-γ-secreting events among 10^6 PBL minus the corresponding numbers of events per 10^6 PBL without antigen. Negative results (spot number without antigen exceeding spot number with antigen) were defined zero. Grey bars, mean response ± SD; black bars, mean positive response ± SD; crosses, median positive response; MG, myasthenia gravis patients; HD, healthy donors. Note the high standard deviations due to heterogeneous responses of single individuals (see Table 1). Note the different Y axis scales using four different recombinant fragments. (a) fragment Alpha 1-103; (b) Alpha 1-209; (c) Alpha 327-398; (d) Epsilon 1-221.

2. Patients and Methods

2.1. Patients and Controls. Peripheral blood lymphocytes (PBL) were obtained with informed consent from patients with generalized or ocular MG (n=15) or healthy donors (HD, n=9). PBL were isolated by density centrifugation and were either frozen immediately and thawed for analysis or used directly.

2.2. Synthesis of Recombinant Fragments. Human α and ε subunit polypeptides were synthesized by PCR on cDNA prepared from total RNA of human calf muscle as described elsewhere [12]. Recombinant protein fragments were kindly provided by Wolfgang Wienhold and Arthur Melms [13]. Fragments were expressed in *E. coli* and purified by SDS/PAGE with a standard protocol [5, 12]. Alpha 1-103 and Alpha 1-209 are fragments of the extracellular domain, Alpha 327-298 of the intracellular domain of the α subunit [1]. Epsilon 1-221 is a fragment of the extracellular domain of the ε subunit.

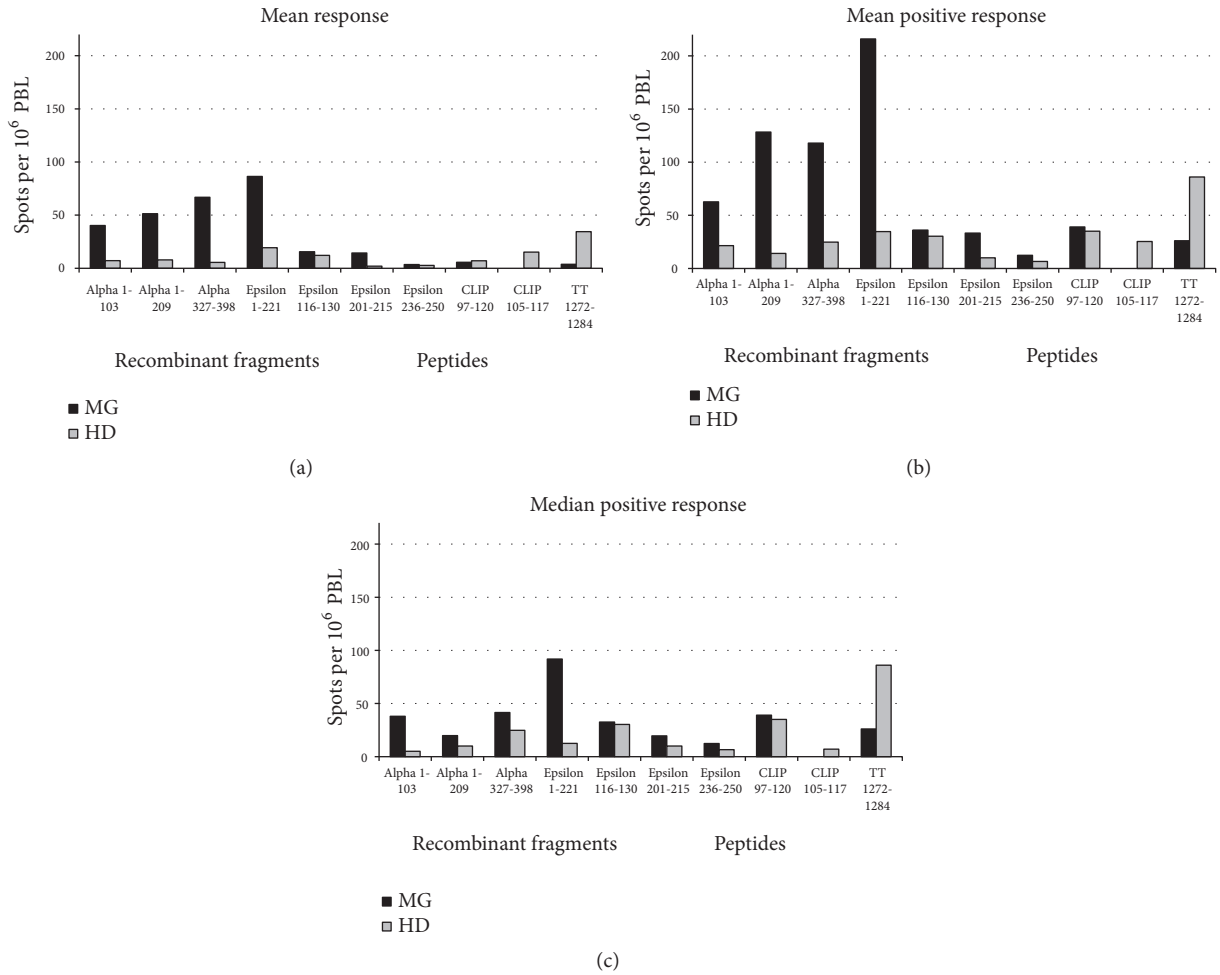

Figure 2: (a) Mean response, (b) mean positive response, and (c) median response of the numbers of IFN-γ-secreting events among 10^6 PBL minus the corresponding numbers of events per 10^6 PBL without antigen. Negative results (spot number without antigen exceeding spot number with antigen) were defined zero. MG, myasthenia gravis patients; HD, healthy donors.

2.3. Synthesis of Peptides. Peptides were synthesized by solid-phase Fmoc-chemistry on an automated peptide synthesizer for multiple peptide synthesis as described previously [14]. Epsilon 116-130, IDGQFGVAYDANVLV, is an HLA-DR3-binding peptide. Epsilon 201-215, ENGEWAIDFCPGVIR, contains a dominant epitope restricted by HLA-DR52a [7]. Epsilon 236-250, IRRKPLFYVINIIVP, contains a dominant T-cell epitope that is not HLA-DR3-restricted. As a specificity control peptide, we used the class II-associated invariant chain peptides, CLIP 97-120, LPKPPKPVSKMRMATPLLMQALPM, and CLIP 105-117, SKMRMATPLLMQA. The tetanus toxoid peptide TT 1272-1284 is a promiscuitive HLA-DR3/DR52a binder.

2.4. ELISPOT Assay. We measured frequencies of interferon (IFN)-γ-secreting T-cells using an ELISPOT (enzyme-linked immunospot) assay as described previously [15]. Microtiter filter plates (Millipore) were coated overnight with an anti-human IFN-γ monoclonal antibody (mAb) (clone 1-D1K; 10 μg/ml, Mabtech, Sweden). After washing and blocking the plates with culture medium (RPMI 1640 supplemented with 5% fetal bovine serum and antibiotics, all from Gibco), fresh or freshly thawed PBL from MG patients and HD were incubated for 20 h in duplicate in the presence or absence of human AChR antigens or controls (1 μg/ml). Concanavalin A (5 μg/ml; Sigma) was used as positive control. Using biotinylated anti-human IFN-γ mAb (clone 7-B61; Mabtech), streptavidin-alkaline phosphatase (Mabtech), and BCIP/NBT as substrate (Sigma), antigen-specific IFN-γ secreting T lymphocytes were visualized and counted on a dissecting microscope. Results are calculated and assigned as the numbers of IFN-γ-secreting events among 10^6 PBL minus the corresponding numbers of events per 10^6 PBL without antigen.

2.5. Statistical Analysis. Student's t-test was performed for statistical analysis. A p value of < 0.05 was accepted to be significant.

3. Results and Discussion

The T-cell responses were heterogeneous throughout MG patients. While single individuals did not show any detectable IFN-γ secreting T-cells after stimulation with AChR fragments (Table 1), others exhibited marked responses (Figures 1 and 2). The responses did not correlate with the

Table 1

Group	Donor	Sex (f/m)	Age (years)	AChR-Ab (nmol/l)	Spots per 10^6 PBL									
						Recombinant fragments				Peptides				
					No antigen	Alpha 1-103	Alpha 1-209	Alpha 327-398	Epsilon 1-221	Epsilon 116-130	Epsilon 201-215	Epsilon 236-250	CLIP 97-120	CLIP 105-117	TT 1272-1284
MG	MG-1	f	70	135.6	1	0	0	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	MG-2	f	62	0.5	11	0	0	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	MG-3	m	48	10.2	2	160	560	416	680	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	MG-4	m	66	0.0	10	0	0	36	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	MG-5	m	50	2.2	5	151	160	320	409	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	MG-6	m	64	0.4	12	2	0	28	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	MG-7	f	66	0.0	38	39	0	5	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	MG-8	m	71	0.0	2	88	20	10	158	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	MG-9	m	58	2.8	13	5	5	0	13	0	0	0	0	0	0
	MG-10	f	82	8.1	6	18	5	0	0	0	3	5	0	0	0
	MG-11	f	25	76.9	20	18	0	13	0	0	0	0	0	0	0
	MG-12	f	36	1.7	39	0	0	0	0	0	0	0	0	0	0
	MG-13	f	53	1.5	1	13	20	20	26	33	20	20	39	0	26
	MG-14	f	38	46.0	3	0	0	0	11	43	0	0	0	0	0
	MG-15	f	32	22.2	134	111	0	156	0	33	78	0	0	0	0
Mean response			**54.7**	**20.5**	**19.8**	**40.2**	**51.3**	**66.8**	**86.4**	**15.5**	**14.3**	**3.5**	**5.6**	**0.0**	**3.7**
SD			**16.4**	**38.5**	**33.9**	**57.5**	**146.5**	**129.7**	**197.0**	**19.6**	**29.0**	**7.3**	**14.7**	**0.0**	**9.8**
Mean positive response						**62.7**	**128.3**	**118.0**	**216.0**	**36.1**	**33.3**	**12.3**	**39.0**	**0.0**	**26.0**
SD						**60.7**	**219.7**	**150.9**	**274.1**	**6.2**	**39.6**	**10.3**			
Median positive response						**38.0**	**19.8**	**41.5**	**91.8**	**32.5**	**19.5**	**12.3**	**39.0**	**0.0**	**26.0**
HD	HD-1	m	47	n.d.	2	0	5	10	13	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	HD-2	f	26	n.d.	3	0	10	0	3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	HD-3	f	30	n.d.	1	0	0	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	HD-4	f	32	n.d.	19	56	0	39	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	HD-5	m	79	n.d.	4	5	3	0	13	0	0	3	0	0	0
	HD-6	f	64	n.d.	49	0	43	0	135	50	10	0	35	0	165
	HD-7	f	53	n.d.	1	4	11	0	11	11	0	11	0	7	7
	HD-8	f	48	n.d.	6	0	0	0	0	0	0	0	0	6	0
	HD-9	m	60	n.d.	95	0	0	0	0	0	0	0	0	63	0
Mean response			**48.8**		**20.0**	**7.1**	**7.9**	**5.5**	**19.3**	**12.1**	**2.0**	**2.6**	**7.0**	**15.2**	**34.4**
SD			**17.5**		**32.1**	**18.3**	**13.7**	**13.1**	**43.8**	**21.7**	**4.5**	**4.5**	**15.7**	**26.7**	**73.1**
Mean positive response						**21.4**	**14.1**	**24.8**	**34.7**	**30.3**	**10.0**	**6.5**	**35.0**	**25.3**	**86.0**
SD						**29.8**	**16.2**	**20.3**	**56.2**	**27.9**		**5.7**		**32.2**	**111.7**
Median positive response						**5.0**	**10.0**	**24.8**	**12.5**	**30.3**	**10.0**	**6.5**	**35.0**	**7.0**	**86.0**

ELISPOT assay was performed as described in the text. Results are assigned as the numbers of IFN-gamma-secreting events among 10^6 PBL minus the corresponding numbers of events per 10^6 PBL without antigen. Negative results (spot number without antigen exceeding spot number with antigen) were defined zero. MG, myasthenia gravis patients; HD, healthy donors; n.d., not done.

AChR-antibody status. For example, patient MG-8 was AChR-antibody negative but exhibited a T-cell response to the AChR protein fragments. Accordingly, some HD presumably AChR-antibody negative gave positive T-cell responses. Analyzing the mean responses of all donors, the mean of detectable (positive) responses only, or the median positive responses only, recombinant fragments of both the α and the ε subunit induced a higher T-cell response in MG than in HD (Table 1). However, statistical analysis could only determine a trend and not statistical significance.

The fragment Epsilon 1-221 showed the highest response. Synthetic peptides of the ε subunit induced a lower response. The most remarkable difference between MG and HD was observed with Epsilon 201-215 containing a dominant T-cell epitope (Table 1) [7]. Consistent with this finding, Ragheb and colleagues have demonstrated proliferative T-cell responses upon stimulation with synthetic peptides of the ε subunit in up to 15% of MG patients including Epsilon 194-209 [2].

Correlations between AChR-specific T-cell responses and paraclinical data (sex, age, or anti-AChR antibody serum titer) were not observed (see Table 1). In an animal model of MG, experimental autoimmune myasthenia gravis (EAMG), Gaertner et al. investigated the pathogenicity of T-cell determinants of the ε subunit [8]. Although IFN-γ secretion by T-cells reactive to ε subunit peptides was observed, these cells failed to induce EAMG upon transfer. Hence, these T lymphocytes were demonstrated to be nonpathogenic. It remains to be determined if this observation reflects a peculiarity of the EAMG model or if nonpathogenic, ε subunit-specific T lymphocytes are present in MG patients. If so, it is speculated that they may contribute to the integrity of the neuromuscular junction [8].

We conclude that the IFN-γ ELISPOT method may provide a valuable tool to measure AChR-specific T-cell responses in MG. In MG patients who tested positive, T lymphocytes specific for epitopes of the AChR ε subunit may be a target for therapeutical intervention in MG.

Data Availability

Data supporting the results of this study can be provided by the corresponding author.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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