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Complementary strategies to elucidate T helper cell epitopes in myasthenia gravis

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Abstract

$CD4^+$ T cells specific for the acetylcholine receptor (AChR) are assumed to play an important role in pathogenesis of myasthenia gravis (MG). A large and diverse number of potential T cell epitopes have been reported for different experimental setups aiming at the identification of disease-relevant T cells in MG. Investigating the T cell response to the epsilon subunit of human AChR, we explore complementary in vitro and in vivo approaches (PBMC from MG patients and mice transgenic for HLA-DR3 and human CD4) to address the possibilities and limitations of different strategies for elucidating natural autoimmune T cell epitopes.

Keywords: Myasthenia gravis; Acetylcholine receptor; T cell epitopes

1. Introduction

Myasthenia gravis (MG) is an autoimmune disorder mediated by pathogenic antibodies against the acetylcholine receptor (AChR) or other components of the muscle end plate. These impair neuromuscular transmission and cause damage to the neuromuscular junction (Vincent, 2002). In addition to the obvious role of B cells, T cells are also considered to be important for disease development, as T cell help is required for generation of high affinity IgG antibodies responsible for the symptoms observed in MG (Conti-Fine et al., 2006). Furthermore, mice deficient in $CD4^+$ T cells are resistant to the experimental form of MG (EAMG) (Kaul et al., 1994). MG is often associated with thymic abnormalities (Hohlfeld and Wekerle, 1994) and thymectomy benefits at least some patients, especially patients with an early onset of disease (Vincent et al., 1983). Thymi of MG patients show an increased expression of the lysosomal protease cathepsin (Cat) V (Tolosa et al., 2003). Cathepsin V is involved in MHC II antigen and invariant chain (Ii) processing, and HLA-DR3 as well as a polymorphism of CatV are associated with early onset MG (EOMG, < 40years at onset) (Price et al., 1999; Viken et al., 2007). T cell lines specific for AChR could be isolated from blood and thymus (Hohlfeld et al., 1984; Sommer et al., 1991; Melms et al., 1992; Melms et al., 1988; Protti et al., 1993) and in an experimental setting, anti-CD4 treatment resulted in clinical improvement (Ahlberg et al., 1994), further underscoring the importance of T cells for the disease.

While the majority of studies have focussed on the α-subunit of the $\alpha_2\beta\delta\varepsilon$ complex which forms the adult form of the receptor, the other subunits can also be used to induce the experimental form of MG (EAMG) in susceptible animals. Hill and coworkers obtained T cell clones specific for the ε rather

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than the α subunit from EOMG patients (Hill et al., 1999). While expression of all subunits could be detected in MG thymi, RNA expression of the ε subunit was increased in about 50% of MG thymi, in accordance with the hypothesis that autosensitization in MG might occur in the thymus (MacLennan et al., 1998; Wekerle et al., 1981).

In order to understand which T cell epitopes might be involved in priming of autoreactive T cells against AChRε we used two complementary approaches. First, we screened T cell responses in peripheral blood of MG patients using a peptide array covering the extracellular domain of the human AchRε subunit. Secondly, we addressed the question of immunodominance and epitope selection in vivo, using an HLA-DR3/human CD4 transgenic mouse lacking endogenous mouse CD4 immunized with AchRε-polypeptide which requires lysosomal antigen processing in antigen-presenting cells. Immunodominance and epitope selection can be examined in the context of HLA-DR3(17) which is associated with early onset of MG and other autoimmune diseases in humans. Interestingly, the two approaches lead to different results with respect to epitope sequences, suggesting that the commonly applied in vitro peptide screening approach, although potentially useful in identifying T cell epitopes, might sometimes be misleading in trying to pinpoint disease-associated epitopes processed and presented in vivo.

2. Materials and methods

2.1. Peptides and protein antigens

The peptide array consisted of 44 pentadecapeptides (ten amino acids overlap) spanning the first extracellular domain and part of the first transmembrane domain of AChRε (amino acids 1–230). The peptides were synthesized and analyzed by HPLC and ESI-MS by EMC microcollections GmbH (Tübingen, Germany). Recombinant human AChRε was generated as described (Gaertner et al., 2004; Melms et al., 1992). Briefly, mRNA coding for AChRε was isolated from human muscle, reverse transcribed and cloned into pBluescipt® II KS+ (Stratagene, USA). The sequences of two constructs AChRε1–255 and AChRε1–221 were subcloned into the bacterial expression vector pQE30 (Qiagen, Germany) containing a 6×His-tag coding sequence. The N-terminally tagged polypeptides were expressed in *E.coli* M15 and purified under denaturing conditions using nickel-nitirilotriacetic acid (Ni-NTA) sepharose columns (Qiagen, Germany). The purity of the recombinant proteins was confirmed by SDS/PAGE analysis. Torpedo AChR was prepared from the electric organ of the electric ray *Torpedo californica* (Melms et al., 1988). AChRα 1–437 and invariant chain 73–232 were generated as described previously (Malcherek et al., 1998; Melms et al., 1992).

2.2. Samples and proliferation assays

Blood was drawn from healthy donors and 57 randomly selected patients (Supplementary Table 1) with a well-documented history of MG after informed consent and PBMC were isolated on Lymphoprep gradients (Nygaard, Norway). 2×10^5 PBMC were cultivated in RPMI containing 3% human serum (male donor, blood group AB), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 20 mM Hepes with or without peptide antigen (10 μg/ml) or protein antigen (1 μg/ml) in a final volume of 100 μl at 37°C. T cell responses to each individual peptide antigen were assessed in quadruplicates. The spontaneous proliferation of PBMC from each subject was assessed in the absence of antigen (12 replicates). The mitogen PHA (5 μg/ml) was used as a positive control for proliferation. After 6 days of culture 7.5 kBq/well ^{3}H-thymidine (Amersham, Germany) per well was added and cells were harvested 16h after labelling. ^{3}H-thymidine incorporation was counted on a Microbeta scintillation counter (Wallac ADL, Finland). Stimulation indexes (SI) were calculated by dividing cpm in the presence of antigen by cpm in the absence of antigen.

The triple DR/huCD4$^+$ transgenic mice have been described before (Laub et al., 2000). For assessment of T cell responses to AChRε antigen, mice were immunized intraperitoneally with 50 μg of recombinant AChRε$_{1-221}$ protein in CFA. Control animals were injected with CFA alone. After 2 days, 10 days, 3 weeks and 4 weeks pertussis toxin (200 ng/mouse, Gibco) was injected. Ten weeks after immunization each animal was challenged with 50 μg of AChRε$_{1-221}$ in CFA. The animals were sacrificed 10 days later. Spleens were removed under sterile conditions and smashed with a syringe pistil. Erythrocytes were lysed by incubation with ACK buffer. Splenocytes were washed and placed in culture in X-Vivo medium (BioWhittacker, USA) containing 50 μM β-mercapto-ethanol, penicillin, streptomycin and L-glutamine. T cell proliferation was assessed by incubating splenocytes in the presence of protein antigen (10 μg/ml–0.01 μg/ml) in triplicate wells. After 48 h the cells were pulsed with ^{3}H-thymidine and harvested 16h later.

Table 1
Sequence and amino acid positions of AChRε peptides used in the study (15-mers in 5 AA steps 10 AA overlap; leader sequence excluded)

1–15	KNEELRLYHHLFNNY	111–125	YEGGSVTWLPPAIYR
6–20	RLYHHLFNNYDPGSR	116–130	VTWLPPAIYRSVCAV
11–25	LFNNYDPGSRPVREP	121–135	PAIYRSVCAVEVTYF
16–30	DPGSRPVREPEDTVT	126–140	SVCAVEVTYFPFDWQ
21–35	PVREPEDTVTISLKV	131–145	EVTYFPFDWQNCSLI
26–40	EDTVTISLKVTLTNL	136–150	PFDWQNCSLIFRSQT
31–45	ISLKVTLTNLISLNE	141–155	NCSLIFRSQTYNAEE
36–50	TLTNLISLNEKEETL	146–160	FRSQTYNAEEVEFTF
41–55	ISLNEKEETLTTSVW	151–165	YNAEEVEFTFAVDND
46–60	KEETLTTSVWIGIDW	156–170	VEFTFAVDNDGKTIN
51–65	TTSVWIGIDWQDYRL	161–175	AVDNDGKTINKIDID
56–70	IGIDWQDYRLNYSKD	166–180	GKTINKIDIDTEAYT
61–75	QDYRLNYSKDDFGGI	171–185	KIDIDTEAYTENGEW
66–80	NYSKDDFGGIETLRV	176–190	TEAYTENGEWAIDFC
71–85	DFGGIETLRVPSELV	181–195	ENGEWAIDFCPGVIR
76–90	ETLRVPSELVWLPEI	186–200	AIDFCPGVIRRHHGG
81–95	PSELVWLPEIVLENN	191–205	PGVIRRHHGGATDGP
86–100	WLPEIVLENNIDGQF	201–215	ATDGPGETDVIYSLI
91–105	VLENNIDGQFGVAYD	196–210	RHHGGATDGPGETDV
96–110	IDGQFGVAYDANVLV	206–220	GETDVIYSLIIRRKP
101–115	GVAYDANVLVYEGGS	211–225	IYSLIIRRKPLFYVI
106–120	ANVLVYEGGSVTWLP	216–230	IRRKPLFYVINIIVP