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No auto-antibodies against synaptic ErbB in seronegative myasthenia gravis

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Abstract

In myasthenia gravis (MG), the neuromuscular transmission is impaired mainly by auto-antibodies against the acetylcholine receptor (AChR). In about 5% of the MG patients, however, the auto-antigen is still unknown. We investigated whether these seronegative MG patients (SNMG) have auto-antibodies against ErbB proteins, which influence the AChR density at the NMJ. Using Fluorescence-activated cell sorting (FACS) and dot blotting analysis of membrane extracts from transfected human embryonic kidney (HEK) cells, we were unable to detect anti-ErbB antibodies in sera of SNMG patients. The results do not support a role for ErbB receptors as auto-antigen in SNMG.

Introduction

Myasthenia gravis (MG) is an acquired autoimmune disease that affects the synaptic transmission between motor neurons and skeletal muscle cells. In about 85% of generalised MG patients, autoantibodies are present against the acetylcholine receptor (AChR), which is located at the postsynaptic membrane of the neuromuscular junction (NMJ). [9] In about 5% of the generalised MG patients the muscle-specific kinase (MuSK) is the target of the autoantibodies while antibodies to AChR are absent. [54] The remaining patients with generalised MG without detectable autoantibodies show symptoms that are similar to those presenting with anti-AChR positive MG. [134] These seronegative MG (SNMG) patients improve after immunosuppressive treatment, which strongly suggests that SNMG also has an autoimmune origin. [111,135] Approximately half of the SNMG subgroup appears to have antibodies to the AChR as well, although probably only with low affinity. [136] In a study by Leite *et al.* anti-AChR antibodies could only be detected by assays where the AChRs were clustered on the cell surface instead of being in a solubilised form, as is the case in routine assays for anti-AChR antibodies. [44] However, in about 5% of MG patients the auto-immune target remains unknown. We therefore tested the possibility that ErbB proteins can act as auto-antigens in SNMG.

ErbB proteins are receptors for neuregulins and are involved in the local activation of synaptic gene expression, such as AChR. [137,138] The ErbB receptor family consists of four members: ErbB1 (also known as the epidermal growth factor receptor or HER1), ErbB2 (also known as HER2 in humans and Neu in rodents), ErbB3 (or HER3) and ErbB4 (or HER4). In skeletal muscle, ErbB3 is expressed mainly at the terminal Schwann cells, whereas ErbB2 and ErbB4 are located postsynaptically in the depth of the folds. [137,139,140] Mutant mice lacking neuregulin or ErbB genes die early in embryogenesis due to defects in cardiac and neural development, but hemizygous mice are viable. [141,142] Mice hemizygous for the neuregulin-1 gene have a reduced concentration of AChRs and show myasthenic symptoms [143,144], while mice hemizygous for the ErbB2 or ErbB3 gene show modest but significant postsynaptic defects, but no clear cardiac defects. [145] As transmembrane proteins, ErbB receptors are potentially accessible to antibodies and could act as autoantigens in an antibody-mediated immune response. Therefore, we studied the presence of antibodies to ErbB receptors in patients with myasthenia gravis without antibodies to AChR or MuSK.

Materials and methods

Patient selection

Patients with generalised MG without anti-AChR antibodies were included from a nationwide study as described previously. [115] The diagnosis of generalised MG was based on acquired and clinically confirmed fluctuating weakness of muscles not restricted to the external eye muscles, the m. levator palpebrae or the m. orbicularis oculi. Absence of antibodies to the AChR in standard radioimmunoassays (RIA) was required more than once during periods of clinical weakness. The diagnosis was considered to be supported by a clinical response to intravenous acetylcholinesterase inhibitors or by electromyography if a decrement of the compound muscle action potential of >10% was found during repetitive nerve stimulation or if single-fiber electromyography had shown an increased jitter or blocking.

All patients were re-examined by a single investigator (EHN) for standardised history taking and physical examination. The clinical condition during the first two weeks and at maximum disease severity was classified according to the MGFA Clinical Classification. [113] Serum from all patients was retested for antibodies to the AChR, MuSK and voltage-gated calcium channels in standard RIA (RSR Ltd., UK). DNA of SNMG patients was tested for the rapsyn N88K mutation known to cause a late-onset congenital myasthenic syndrome. [45] Of the 62 patients with SNMG originally described in our epidemiological study, seven were lost to follow-up, while no informed consent was obtained from six, one patient had died and one 16-year-old girl had become AChR antibody positive three years after the onset of the symptoms. The remaining 47 patients were clinically re-examined. Subsequently, five patients were excluded because the symptoms were incompatible with generalised MG (n=2), or because of the presence of a N88K rapsyn mutation (n=1), or comorbidity in the form of severe Cushing's disease before the onset of MG symptoms (n=1), or finally because one patient appeared to have presented fluctuating weakness ever since birth (classified as congenital myasthenic syndrome, n=1). Clinical characteristics of 42 patients with generalised SNMG are summarised in Table 6.1. Muscle weakness was mild or moderate, and more of the limb-girdle type (MGFA class IIa or IIIa). However, a few patients suffered from oculobulbar and axial weakness, even requiring intubation in two cases (MGFA class V). The diagnosis was supported by repetitive nerve stimulation in 14 patients, by single-fiber electromyography in 17 patients and by IV acetylcholinesterase inhibitor in eight patients. In one patient, diagnostic procedures were incomplete. Only repetitive nerve stimulation was performed but negative. In the remaining two patients, results of repetitive nerve stimulation, single-fiber electromyography and IV acetylcholinesterase inhibitor were all negative. In all three patients however, clinical symptoms had responded to immunosuppressive therapy.

Table 6.1 Characteristics of 42 SNMG patients

Women - *n* (%)	28	(67)
Age at onset - median (range) in yrs	38.1	(5.9 - 66.8)
Age at onset below 16 yrs - *n* (%)	5	(12)
Follow-up - median (range) in yrs	10.4	(0.6 - 45.5)
Caucasian - *n* (%)	40	(95)
MGFA at onset		
- I - *n*	19	
- II (a *vs*. b) - *n*	23	(14 *vs*. 6)
MGFA at maximum or before thymectomy for French patients		
- II (a *vs*. b) - *n*	20	(11 *vs*. 2)
- III (a *vs*. b) - *n*	15	(9 *vs*. 4)
- IV (a *vs*. b) - *n*	3	(0 *vs*. 2)
- V - *n*	2	
Interval onset to maximum - median (range) in yrs	2.1	(0 - 40.5)
Diagnosis supported by		
- A. Electromyography - *n* (%)	31	(74)
- B. Response to IV AChE inhibitors - *n* (%)	8	(19)
- Neither A or B - *n* (%)	3	(7)
Therapy		
- Oral AChE inhibitors - *n* (%)	41	(98)
- Immunomodulative therapy - *n* (%)	26	(62)
- Oral immunosuppression - *n* (%)	23	(55)
- Thymectomy (with follicular hyperplasia) - *n*	11	(3)
Interval onset to start of immunomodulation - median (range) in yrs	2.4	(0 - 40.5)

Fluorescence-activated cell sorting (FACS)

Sera from 42 SNMG patients were screened for anti-ErbB-IgG using FACS. For this purpose murine 32D hematopoietic cells transfected with distinct combinations of human ErbB receptors were used. In the present study we used 32D13 cells (expressing both ErbB1 and ErbB3), 32D23 cells (expressing both ErbB2 and ErbB3) and 32D24 cells (expressing both ErbB2 and ErbB4). Transfected 32D cells were cultured in RPMI 1640/glutamax-I, containing penicillin (100 U/ml), streptomycin (100 µg/ml), 10% fetal calf serum (FCS), 50 µM β–mercaptoethanol, recombinant mouse IL-3 (0.5 ng/ml, R&D systems, Minneapolis, MN, USA), geneticin (G418, 600 µg/ml) and hygromycin B (for 32D13 and 32D23 cells, 400 µg/ml). The cells were cultured in a 37°C humidified atmosphere containing 95% air and 5% CO2. High density 32D cells were harvested and resuspended in washing buffer (PBS containing 1% BSA/1% FCS/0.2% NaN3). Subsequently cells (0.15x106) were incubated with sera diluted to a final concentration of 1:10, 1:20 and 1:40 in washing buffer for 1 hour while gently shaking. Cells were then washed five times with washing buffer and incubated for 30 min with rabbit anti-human-IgG-FITC conjugate (1:20, Dako, Glostrup, Denmark). As a negative control, normal human serum (NHS; healthy volunteers) was

analysed according to the same protocol. The expression of ErbB2 was checked with a mouse monoclonal anti-c-ErbB2 (clone L26, Immunologic, Duiven, The Netherlands) and goat anti-mouse Ig-APC conjugate (BD Pharmingen, San Jose, CA, USA). Cell lines 32D23 and 32D24 were positive for ErbB2 staining in contrast to 32D13 which was negative. FACS analysis was performed using a BD Biosciences FacsCalibur flowcytometer and the mean fluorescence intensity (MFI) was calculated with CellQuestPro software (BD Biosciences, San Jose, CA, USA). The MFI of each sample was normalised by dividing the obtained fluorescence values by the mean of the corresponding NHS group. The mean value of NHS plus two and three times the standard deviation was used as cut off value.

Dot blotting

Sera from 42 SNMG patients were also screened for anti-ErbB-IgG using dot blotting. For this, we used human embryonic kidney (HEK) 293 cells, transiently transfected with expression vectors for human ErbB2 and ErbB4 genes using the Profection mammalian calcium phosphate transfection system (Promega Corporation, USA). These vectors were a kind gift of Dr. L. Schaeffer from the 'Laboratoire de biologie moleculaire et cellulaire' in Lyon, France. Transfected cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with glucose and sodium pyruvate (Gibco, Invitrogen Corporation, Breda, The Netherlands) supplemented with 10% FCS (Bodinco, The Netherlands), 1% penicillin/streptomycin (Gibco) and 1% L-glutamine (Gibco). The cells were cultured in a 37°C humidified atmosphere containing 95% air and 5% CO2. After 72 hours, membrane proteins were isolated from the cells with a 0.6% cholate extraction buffer, containing 0.01 M NaN3 (Acros Organics, Geel, Belgium), 0.01 M EDTA (Sigma-Aldrich, St-Louis, USA), 0.01 M iodoacetamide (Sigma), 0.1 mM phenylmethylsulphonyl fluoride (Sigma) and 0.6% cholate (Sigma) in PBS. The protein concentration was measured using the Bio-Rad system (Bio-Rad, California, USA). To verify the expression of ErbB2 and ErbB4 in transfected HEK cells, Western blotting was performed as described before using rabbit anti-ErbB2 antibody (C-18) (sc-284, Santa Cruz Biotechnology Inc., USA) or of rabbit anti-ErbB4 antibody (C-18) (sc-283, Santa Cruz).[146]

To detect anti-ErbB antibodies in sera of SNMG patients, 5 µl of ErbB transfected HEK 293 cell extract (0.4 mg/ml protein concentration) and non-transfected cell extract (0.4 mg/ml) were applied in three dots on a Protran nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). Following blocking in Odyssey blocking buffer (OBB; LiCor Westburg, Leusden, The Netherlands) diluted in PBS (1:1) (OBB/PBS), membrane strips were separately incubated with rabbit anti-ErbB2-antibody or anti-ErbB4 antibody (positive controls), diluted 1:1000 or 1:1500 respectively in OBB/PBS containing 0.5% goat serum (GS) (i.e. OBB/PBS/GS), with OBB/PBS/GS (negative control), with patient serum diluted 1:1000 in OBB/PBS/GS, or with NHS diluted 1:1000 in OBB/PBS/GS. Then, strips were

washed three times with PBS 0.1% Tween (Merck), and incubated with IRDye 800 goat anti-rabbit IgG (Rockland Immunochemicals), diluted 1:3700 in OBB/PBS/GS, or with IRDye 800 goat anti-human IgG (Rockland Immunochemicals), diluted 1:10000. Following the washing steps, strips were scanned with the Odyssey scanner (LiCor Westburg) at 800 nm. Finally, ImageJ software (http://rsb.info.nih.gov/ij/) was used to quantify the integrated intensities of the dots. The mean integrated intensity of the background was subtracted. To correct for the non-specific binding of antibodies to other proteins than ErbB2 or ErbB4, the ratio was calculated between mean intensities of dots of transfected cell extract and dots of non-transfected cell extract. These ratios were normalised by dividing them by the mean of the NHS ratios. The mean of NHS plus two and three times standard deviation was used as cut off value.

Statistical analysis

SPSS Software 17.0 was used to perform statistical analyses. Comparison between non-normally distributed values was performed using the non-parametric Mann-Whitney test. A two-sided probability value <0.05 was considered statistically significant.

Results

Results of the anti-ErbB antibody measurement by FACS are summarised in Figure 6.1. The median MFI of SNMG patients was not significantly higher than that of healthy controls, for none of the three cell lines tested. In addition, no individual value of an SNMG patient or a healthy control exceeded the cut off value, calculated as the mean plus three times SD of the corresponding NHS group (data not shown). These results show that there are no detectable levels of antibodies against ErbB1, ErbB2, ErbB3 and ErbB4 in this cohort of SNMG patients.

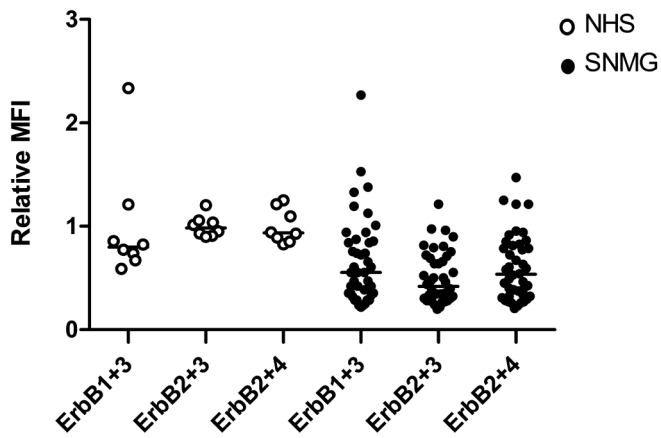

Figure 6.1 Detection of anti-ErbB2/3/4 antibodies by FACS
Sera from 42 SNMG patients (SNMG) were screened for anti-ErbB-IgG by flow cytometry. 32D13 cells (expressing ErbB1+3); 32D23 cells (expressing ErbB2+3); and 32D24 cells (expressing ErbB2+B4) were incubated with serum from patients (solid circles) or normal human serum (NHS) (empty circles). Values are normalised with the mean MFI of the corresponding NHS group. Horizontal full lines represent the median MFI per group. The Mann-Whitney test did not show a significant increase in median MFI in SNMG patients compared to NHS.

Results of the dot blotting analysis are shown in Figure 6.2. The high transfection efficiency of HEK cells with ErbB2 and ErbB4 cDNA was verified by Western blotting (Figure 6.2A). Low amounts of ErbB2 receptors were also endogenously present in non-transfected HEK cells, whereas ErbB4 receptors were undetectable in non-transfected HEK cells. Figure 6.2B shows representative dot blots, where non-transfected and transfected HEK cell extracts were incubated with SNMG patient serum (Figure 6.2B, row 1), normal human serum (Figure 6.2B, row 2), monoclonal anti-ErbB2 antibody (Figure 6.2B, row 3) and monoclonal anti-ErbB4 antibody (Figure 6.2B, row 4). To correct for unspecific antibody binding to endogenous HEK proteins, the mean integrated intensity of transfected cells were divided by the mean integrated intensity of non-transfected cells for each sample. The results are summarised in Figure 6.2C. The median fluorescence ratio in SNMG patients was significantly lower than that of both positive controls (monoclonal anti-ErbB2 antibody ($p<0.001$) and monoclonal anti-ErbB4 antibody ($p<0.001$)), but did not differ from the median ratio of healthy individuals. In addition to comparing median values of the ratio of the different groups, no individual ratio of an SNMG patient or a healthy person exceeded the cut off value.

To study the possibility that the negative results were due to low sensitivity of the techniques, we validated the sensitivity of the dot blotting protocol (Figure 6.2D). Anti-AChR antibodies were measured in AChR-MG patients, of whom the anti-AChR antibody titre had been determined by RIA before. Anti-AChR antibodies were detectable using the present dot blotting technique, although the dot blotting was less sensitive than the RIA (data not shown).

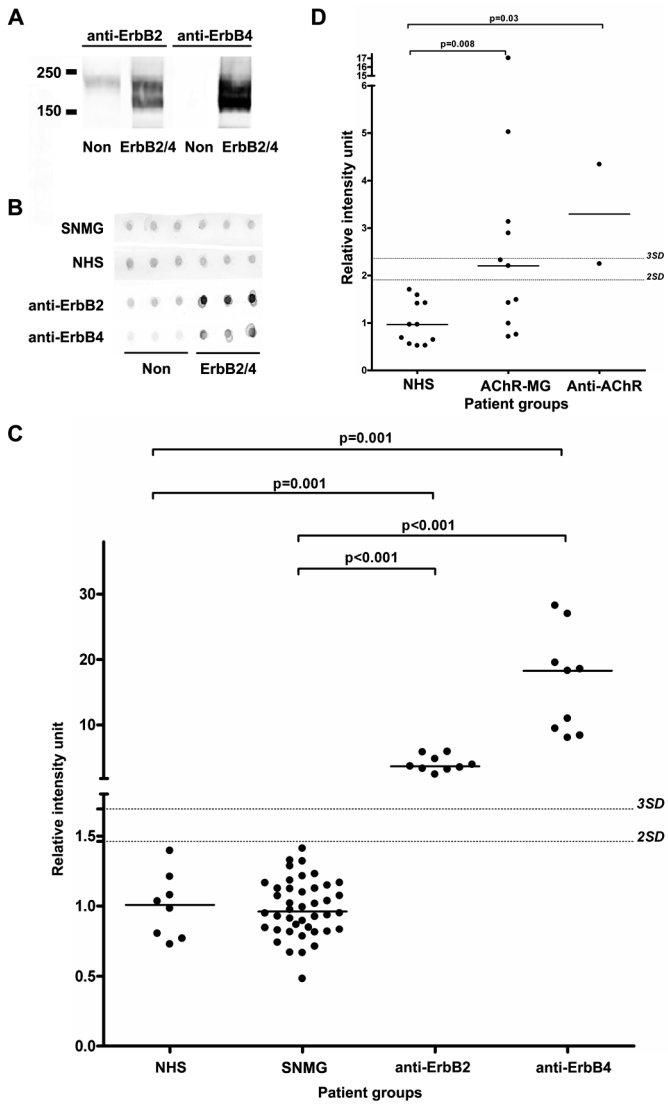

Figure 6.2 Detection of anti-ErbB2/4 antibodies by dot blotting analysis

A) Expression of ErbB2 and ErbB4 in non-transfected (non) and transfected (ErbB2/ErbB4) HEK 293 cells, measured by Western blotting. B) Representative dot blots spotted with non-transfected and transfected HEK 293 cell extracts were incubated with SNMG patient serum (SNMG), normal human serum (NHS), anti-ErbB2 positive control antibody or anti-ErbB4 positive control antibody. C) Dot blot results of anti-ErbB antibody measurement in SNMG patients. Each dot corresponds to the ratio between the mean intensity of triplicate measurements of transfected HEK cells and the mean intensity of the non-transfected HEK cells. Horizontal full lines represent the median value of the ratio per group, dashed lines show cut off value calculated as the mean of NHS plus two and three times the standard deviation. D) Dot blot results of anti-AChR antibody measurement in AChR-MG patients. Horizontal full lines represent the median ratio per group, dashed lines show cut off value calculated as the mean of NHS plus two and three times standard deviation. The *p*-values were obtained by the Mann-Whitney test and shown when <0.05.

Discussion

Various studies have identified ErbB receptors, which are present at the NMJ, as potential candidates for the autoimmune target in SNMG patients, particularly since myasthenic symptoms have been described in ErbB mutant mice. [141-145] The antibody tests for ErbB in the sera of the 42 SNMG patients were all negative. This strongly suggests that ErbB does not have a role as autoantigen in MG.

The present negative finding could be explained if the sensitivity of our assays for anti-ErbB would have been too low. However, we successfully used an analogue FACS analysis to demonstrate the presence of anti-MuSK antibodies, which confirms the sensitivity of the technique (data not shown). The dot blot assay was not able to detect anti-AChR antibodies in all the AChR-MG patients, and thus might be too insensitive for detection of low anti-ErbB antibody titres. A second possibility is that the autoantibodies would only be able to bind to ErbB proteins when clustered together in the NMJ, as shown earlier for a subset of anti-AChR antibodies. [44] A third, more remote, possibility is that patients with a low titre of putative antibodies might bind to the ErbB antigens with very high affinity, thereby leaving the blood serum depleted of free antibody.

If, on the contrary, anti-ErbB antibody would have been present in patients it is questionable that they would actually have caused MG-like symptoms. ErbB2 gene amplification and protein overexpression are known to be critical for the development of malignancies such as breast cancer. For more than ten years, such patients are frequently treated with trastuzumab (Herceptin), a humanised IgG1 monoclonal antibody directed against ErbB2. [147-149] Distinct side effects can occur during treatment with trastuzumab but, as far as we know, myasthenic muscle weakness in patients undergoing this therapy has not been reported, arguing against a possible myasthenic effect of anti-ErbB2 antibodies. No reports specifically analysed these patients for myasthenic syndromes, leaving the possibility that milder forms of the disease might have been missed in these patients.

In conclusion, we could not detect anti-ErbB antibodies in sera of SNMG patients, and therefore further research is needed to identify unknown autoantigens. Interestingly, the thymic transcriptome of SNMG patients is characterised by a down-regulation of several genes involved in synaptic transmission, including neurotransmitter receptors. [150,151] These genes could potentially correspond to autoimmune targets, and would be worthwhile to focus on while seeking other autoantigens responsible for SNMG.

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