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Musculoskeletal Pathology

The Effect of Plasma From Muscle-Specific Tyrosine Kinase Myasthenia Patients on Regenerating Endplates

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Muscle-specific tyrosine kinase (MuSK) is essential for clustering of acetylcholine receptors (AChRs) at embryogenesis and likely also important for maintaining synaptic structure in adult muscle. In 5 to 7% of myasthenia gravis (MG) cases, the patients' blood contains antibodies to MuSK. To investigate the effect of MuSK-MG antibody on synapse regeneration, notexin was used to induce damage to the flexor digitorum brevis muscle. We administered aliquots of MuSK-MG patients' plasma to the flexor digitorum brevis twice daily for a period up to 21 days, and muscles were investigated *ex vivo* in contraction experiments. AChR levels were measured with ^{125}I-α-bungarotoxin, and endplates were studied with quantitative immunohistochemistry. In normal muscles and in 14-day regenerated muscles, MuSK plasma caused impairment of nerve stimulus-induced contraction in the presence of 0.35 and 0.5 mmol/L Ca^{2+} with or without 100 to 400 nmol/L tubocurarine. Endplate size was decreased in regenerated muscles relative to controls; however, we did not observe such differences in muscle not treated with notexin. MuSK plasma had no effect on the amount and turnover rate of AChRs. Our results suggest that anti-MuSK antibodies influence the activity of MuSK molecules without reducing their number, thereby diminishing the size of the endplate and affecting the functioning of AChRs. ***(Am J Pathol 2009, 175:1536–1544; DOI: 10.2353/ajpath.2009.090040)***

Weakness of skeletal muscle in myasthenia gravis (MG) is generally caused by loss of acetylcholine receptors (AChRs) due to circulating autoantibodies against the α-subunit of the AChRs at the motor endplate (AChR-MG).[1–3] These antibodies act mainly through a combination of increased turnover of AChRs, following divalent antibody binding to adjacent AChRs, and complement-mediated attack on the postsynaptic membrane leading to further loss of AChRs and probably other important components of the neuromuscular junction.[3–5] In approximately 15% of the cases with symptoms of MG, autoantibodies to AChRs are absent ("seronegative" MG), although some patients may not be truly seronegative because their sera contain antibodies that bind to concentrated, rapsyn-clustered AChRs expressed in human embryonic kidney cells.[6] Passive immunization of mice with seronegative MG serum causes a reduction of the miniature endplate potentials (MEPP) amplitude, although the number of AChRs is usually not reduced.[7–9]

In many seronegative MG patients with generalized MG, there are antibodies against extracellular regions of the muscle-specific receptor tyrosine kinase, MuSK[10]; in

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central Europe, the proportion is approximately 40%, but in some countries, this fraction may be lower, e.g., 22% in The Netherlands.[11] Most MuSK-MG patients have predominantly bulbar symptoms, often associated with muscle atrophy.[12–14] MuSK antibodies belong predominantly to the IgG4 class,[15,16] which does not activate the classical pathway of the complement system. Furthermore, it is functionally monovalent because IgG4 exchanges Fab arms *in vivo* with nonpathogenic IgG4.[17] Consequently, anti-MuSK IgG4 should not be able to cause a complement-mediated attack on the postsynaptic membrane and may not be able to reduce the number of MuSK molecules by a cross-linking/endocytosis mechanism, because for this functionally, divalent antibodies would be required. Thus, it seems likely, assuming the MuSK antibody is the pathogenic agent responsible for the disease, that antibodies must interfere directly with the physiological function(s) of MuSK.

Most of what we know about the function of MuSK comes from *in vitro* and developmental studies. Studies on cell cultures have revealed that agrin activates the receptor lrp4, which then triggers MuSK to induce the clustering of AChRs during synapse formation through interaction with rapsyn.[18–21] Gene disruptions of agrin, lrp4, MuSK, or rapsyn are lethal because AChRs do not get clustered, and synapses fail to be formed.[18,20,22] It has been suggested that MuSK is essential for maintenance of ultrastructure and anchored AChRs in the adult endplate, because injection and electroporation of dsRNA targeting MuSK causes a reduction of the level of MuSK and disintegration of mouse endplates within 6 weeks.[23] MuSK appears also to have a stabilizing effect on the turnover of AChRs in adult muscle, because exposure of denervated muscles to agrin increases the half-life time of AChRs from 1 to 10 days.[24] Therefore, we hypothesized that the MuSK antibodies that arise in patients after endplates have formed act by interference with the lrp4-agrin-MuSK-rapsyn-AChR pathway, resulting in degeneration of neuromuscular junctions and disturbance of AChR turnover. We explored this idea by testing the effect of MuSK-MG plasma on regenerating instead of normal endplates to see whether under this condition the antibody has an especially pronounced effect. To this end, we first developed a mouse model for regenerating endplates in the flexor digitorum brevis (FDB) muscle based on the reversible myotoxic action of notexin, which is similar to a model earlier described for the soleus muscle of the rat.[25] We found among other things that endplates in regenerated muscles from mice treated with MuSK-MG plasma were smaller than those from mice treated with control plasma and that neuromuscular transmission in these muscles was more sensitive to low Ca^{2+} concentration and tubocurarine.

Materials and Methods

Patients and Blood Plasma

Plasma samples from five MuSK-MG patients were used, four obtained through plasmapheresis. Titers of anti-MuSK antibody, assayed as described earlier,[27] were in the range of 4.6 to 50.4 nmol/L (Table 1). The collected blood plasma was divided into 50-ml aliquots and kept at −20°C until further use. For controls, plasma was used from patients without a neuromuscular disorder, also after plasmapheresis.

Table 1. Anti-MuSK-Positive Patients

Patients	Sex	Age at onset (years)	Severity of disease*	Plasma anti-MuSK$^+$ titre (nM)
1	F	33	IIIb	50.4
2	M	29	IVb	46.2
3	F	29	IIb	12.3
4	F	3	IIIb	4.6
5	F	28	IIb	47.9

*Quantitative MG score.[26]
All patients were treated with prednisone; patient 2 received azathioprine as well.

Five-milliliter aliquots of plasma were dialyzed overnight at 4°C against modified Ringer's solution (M-Ringer), composition: 136 mmol/L NaCl, 4.6 mmol/L KCl, 1 mmol/L $MgSO_4$, 2 mmol/L $CaCl_2$, 1 mmol/L NaH_2PO_4, 2.5 mmol/L $NaHCO_3$, and 11 mmol/L glucose.[28] The dialysis procedure served to standardize the ionic content of the plasma and to wash away the drugs in the plasma, mostly steroids (Table 1). Any traces of drugs remaining would be greatly diluted from the region of injection (see below) by systemic washout (ratio injection volume/body weight was approximately 10^{-3}).

Animals and Muscles

Female Swiss mice (8-week-old) were injected subcutaneously into the foot sole with 0.125 μg notexin (Sigma-Aldrich) dissolved in 25 μl M-Ringer. Mice received similar injections of 40-μl of dialyzed plasma from MuSK-MG or control patients, twice daily for a period of up to 21 days (not during weekends), starting on the day after the notexin injection. For experiments with normal muscle, the mice received plasma up to 14 days. The experimental procedures had been approved by the Animal Ethics Committee at the Leiden University Medical Centre (approval no. 01094), as required by the Dutch Law on Animal Experiments.

Mice were sacrificed by cervical dislocation and the FDB muscles were dissected, together with their nerve branch leading to the sciatic when appropriate.[29] For some experiments, hemidiaphragms were dissected with the phrenic nerve attached.

For contraction experiments, muscles were mounted, bathed at 24 to 28°C in Ringer's medium (116 mmol/L NaCl, 4.5 mmol/L KCl, 1 mmol/L $MgSO_4$, 2 mmol/L $CaCl_2$, 1 mmol/L NaH_2PO_4, 23 mmol/L $NaHCO_3$, and 11 mmol/L glucose, pH 7.4, bubbled with carbogene), and stimulated at the nerve or directly at the muscle as explained elsewhere.[29] Muscles were exposed to tubocurarine and decreased concentrations of calcium.[29]

The AChR binding sites for α-bungarotoxin (α-BuTX) were measured by incubation of muscles for 2 hours in

the presence of 1 μg/ml ^{125}I-α-BuTX in M-Ringer.[28] Excess label was washed away overnight in M-Ringer at 4°C, and subsequently, the muscles were carefully cleaned from all adherent tissue and placed in a gamma scintillation counter. Correction for nonspecific binding of α-BuTX by measuring binding of radioactivity to endplate-free parts of the FDB muscle was not possible,[28] the FDB fibers being only 1–2 mm long, which precluded the division into endplate-containing and endplate-free parts of muscle. However, because the fibers of the FDB are so short, the extrajunctional nonspecific ^{125}I-α-BuTX binding is relatively little compared with, for instance, the diaphragm muscle with a very much larger extrajunctional area.[28] On the basis of our earlier work on the diaphragm, we estimate the nonspecific binding in the FDB to be approximately 10% of the total amount of ^{125}I-α-BuTX bound.

For turnover studies of AChRs, ^{125}I-α-BuTX was injected into the foot sole (40 μl with a total of 5 μg/ml α-BuTX). In each experiment, a correction was made for the radioactive decay of ^{125}I.

Histology and Histochemistry

Cryostat sections (hematoxylin and eosin) of normal and notexin-treated muscles were prepared. For histochemistry, the FDB muscles were quickly frozen in melting isopentane, precooled in liquid nitrogen. Cryosections of 10 μm were cut and stained for AChR (Alexa 594-conjugated α-BuTX) and the vesicular acetylcholine transporter (rabbit anti-VAChT) and analyzed quantitatively as described previously.[30,31] The murine IgGs were blocked with a mouse on mouse (M.O.M.) immunodetection kit (Vector Laboratories, Burlingame, CA) following manufacturer's recommendations. Additionally, mouse anti-rapsyn was used as described,[30,31] with M.O.M.-biotinylated anti-mouse streptavidin Alexa-350 1/500 (Invitrogen). The fluorescence intensities of rapsyn labeling correlate well with histochemical measurements of the AChR in the same endplates (data not shown). Pictures were analyzed using the ImageJ software (version 1.37v; National Institutes of Health, Bethesda, MD). The fluorescence intensities of α-BuTX labeling correlate well with biochemical measurements of the AChR, with the advantage that the histochemical analysis can be confined to the area of the neuromuscular junction without measuring extrajunctional AChR.[30] Endplate areas were identified as regions of vesicular acetylcholine transporter (VAChT) staining and the mean intensity of VAChT and AChR staining was measured in the corresponding area. The threshold intensity of VAChT staining for defining the margin of junctional areas was automatically calculated for each picture. The ratios of AChR:VAChT were calculated as a relative measure for the postsynaptic AChR concentration for 25 to 200 endplates per muscle. All sections were stained and processed in parallel to avoid interassay variations.

Statistics

The data are presented as the mean ± SEM. Possible statistical differences were analyzed with a paired or unpaired Student's *t*-test and two-way analysis of variance wherever appropriate.

Results

A Model for Regenerating Endplates

The supplemental data explain the notexin model that has been used for most experiments in this study. Subcutaneous injection of notexin into the foot sole led to complete destruction of FDB muscle fibers within 2 days, leaving debris and connective tissue (Supplemental Figure S1, see *http://ajp.amjpathol.org*). However, by 15 days after the notexin injection, the muscle fibers had regenerated and the FDB nuclei, which are normally situated peripherally (Supplemental Figure S1A, see *http://ajp.amjpathol.org*), were found in the center of the fiber, characteristic of muscle regeneration (Supplemental Figure S1C, see *http://ajp.amjpathol.org*). This present pattern of notexin-induced degeneration/regeneration of the FDB muscle is similar to that reported earlier for the soleus muscle of the rat after injection of notexin[25] or the myotoxic drug bupivacaine.[32]

By 3 days after notexin treatment and labeling of AChRs by injection of radioactive α-BuTX, most radioactivity was lost from the muscle (Supplemental Figure S1D, see *http://ajp.amjpathol.org*). When notexin-treated muscles were exposed *in vitro* to ^{125}I-α-BuTX, an extra amount of radioactivity was bound starting at day 4 (Supplemental Figure S1D, see *http://ajp.amjpathol.org*). Nerve stimuli-induced contraction of the FDB was first restored also at day 4.

To examine the half-life of the new AChRs, we injected radioactive α-BuTX *in vivo* at various times after the notexin treatment. The half-life of ^{125}I-α-BuTX-labelled AChR in notexin-treated muscle was only approximately 2 days when the toxin was injected just after the new AChRs had formed in the regenerated muscle (Supplemental Figure S1E, see *http://ajp.amjpathol.org*). When the label was injected at 14 days, the half-life time had increased to 5 days. On the other hand, the half-life time was 10 days when the label was given to normal muscle not treated with notexin.

The diaphragm is known to have a great safety factor of neuromuscular transmission, see for instance Ref.[33]. The normal FDB was almost as resistant to paralysis by tubocurarine as the hemidiaphragm (Supplemental Figure S1F, see *http://ajp.amjpathol.org*). Supplemental data S1G (see *http://ajp.amjpathol.org*) show that newly formed fibers in the FDB after notexin treatment were paralyzed at 400 nmol/L tubocurarine to about the same degree as normal muscle.

The Effect of MuSK-MG Plasma

We treated mice with notexin and subsequently injected plasma from MuSK-MG patients into the foot sole for 2

Table 2. Effect of MuSK-MG Plasma on Muscle Contraction in Regenerating FDB Muscles

Medium	Single pulses		40 Hz pulses	
	2 mmol/L Ca^{2+}	0.35 or 0.5 mmol/L Ca^{2+}	2 mmol/L Ca^{2+} 0 nmol/L tubocurarine	2 mmol/L Ca^{2+} 400 nmol/L tubocurarine
MuSK-MG patients, regenerating muscles				
1	118 ± 15 (8)	40 ± 10 (10)*	117 ± 9 (7)	99 ± 8 (8)
2	102 ± 12 (7)	59 ± 13 (8)*	110 ± 13 (7)	97 ± 13 (7)
3	137 (1)	69 (1)	185 (1)	203 (1)
4	111 ± 2 (2)	49 (1)	110 ± 1 (2)	110 ± 20 (2)
5	86 ± 24 (2)	73 ± 8 (2)	81 (1)	123 ± 43 (2)
Mean ± SEM	111 ± 8	58 ± 17**	121 ± 17	126 ± 20

Contractions induced by nerve stimulation are presented as a percentage of matching contractions of muscles from human control plasma-injected mice. The experiments were performed 14 to 16 days after notexin injections with MuSK-MG and control plasma injections starting at day 2. Means ± SEM with the number of tested mice between parentheses. *$P < 0.005$, compared with controls; statistically significant difference compared with values in 2 mmol/L Ca^{2+}, **$P < 0.05$, $n = 5$ patients, Student's paired *t*-tests.

weeks. None of the plasma samples of the patients caused impairment of contraction in the presence of normal physiological solution (with 2 mmol/L Ca^{2+}) on delivery of single pulses to the nerve. It was thought possible that MuSK-MG plasma caused a relatively small decrease in the efficiency of neuromuscular transmission, eg, due to a decrease in the number of AChRs or the amount of ACh released by the nerve impulse. If such a defect would still allow endplate potentials that are still larger than the firing threshold, muscle contraction would still not be affected. Such "subclinical" defects can be made visible by decreasing ACh release by lowering the concentration of calcium in the medium or otherwise by decreasing the number of available AChRs by adding tubocurarine to the bath.

Indeed, when the concentration of Ca^{2+} was decreased from 2 to 0.5 mmol/L or 0.35 mmol/L, plasma samples from five patients caused an approximately twofold, statistically significant, decrease in nerve stimulus-induced muscle contraction relative to muscles derived from mice treated with control plasma (Table 2, $P < 0.05$, $n = 5$ patients). In patients 1 and 2, plasma under the condition of stimulation in 0.35 or 0.5 mmol/L Ca^{2+} caused repeatedly a highly significant decrease of the contraction relative to controls (Table 2, $P < 0.005$, $n =$ 15–18 mice).

When the nerve was stimulated at 40 Hz instead of single pulses, muscles from mice treated with patients' plasma contracted as well as those from control plasma-treated mice in the presence of 2 mmol/L Ca^{2+}. The addition of 400 nmol/L tubocurarine to the medium caused in the muscles from control plasma-treated mice an approximately 20% decrease in nerve stimulus-induced contraction (data not shown). In the presence of 400 nmol/L tubocurarine, MuSK muscles did not show a further reduction in contraction force, relative to controls (Table 2).

For subsequent experiments, we focused on patient 1 plasma because this had the highest anti-MuSK antibody titer and caused the strongest effect on contraction. Figure 1 shows that the nerve stimulus-induced contractions of muscles from MuSK-MG plasma-treated mice as a function of Ca^{2+} concentration, at 14 days after notexin injection, were more affected than the contractions of control muscles. This effect of MuSK plasma must have been due to an effect on neuromuscular transmission per se because the contraction as a result of direct stimulation of the muscle fibers was hardly affected by Ca^{2+} and was not different between MuSK and controls. In other experiments (data not shown), the effect of MuSK plasma was tested already after 7–8 days after notexin injections. The contraction was weak in these muscles, but this was due to the immature condition of the (2- to 3-day-old) fibers rather than to the efficiency of neuromuscular transmission, which appeared already rather good when the nerve stimulus-induced contractions were compared with those resulting from direct muscle stimulation. Because of the weakness of the contractions, it was difficult to ascertain the effect of the MuSK plasma treatment, although the impression was that there was, relative to controls, not a marked effect of MuSK plasma on the contractions in 0.35 mmol/L Ca^{2+}.

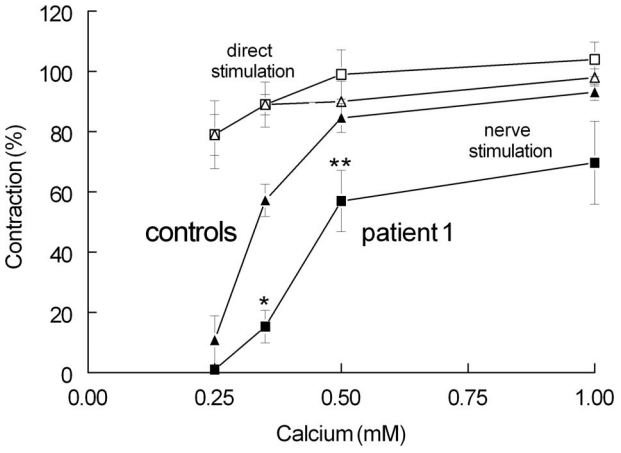

Figure 1. Effect of calcium and MuSK-MG plasma treatment (patient 1) on the contraction of regenerating FDB muscle (14 to 16 days after notexin). Plasma injections were started 1 day after notexin. Nerve stimulation with single pulses in muscles treated with patient's plasma (squares) and control plasma (triangles). Direct stimulation of the same muscles with single pulses (open squares and triangles for patient and control, respectively). Means ± SEM of values from six to eight muscles. *$P < 0.005$; **$P < 0.05$ (Student's *t*-test).

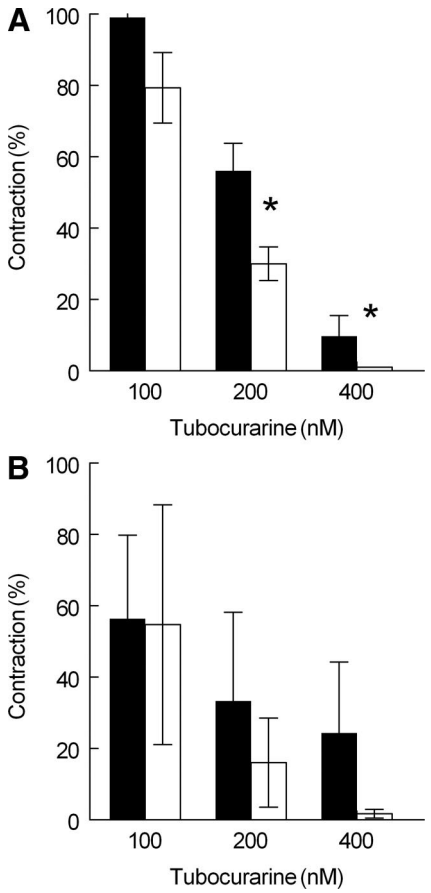

Figure 2. Effect of anti-MuSK$^+$ plasma (patient 1) and tubocurarine on the contraction of normal (**upper panel**) and notexin-treated (14 days, **lower panel**) FDB muscles in the presence of 0.5 mmol/L (**upper panel**) or 0.35 mmol/L Ca^{2+} (**lower panel**). Nerve stimulation at 40 Hz. Ordinate, the contractions of muscles are expressed as a fraction of the contractions caused by direct stimulation of the muscle. Data derived from mice treated with control plasma (black bars) and patient 1 plasma (white bars). Means ± SEM of three muscles. *$P < 0.05$ (ANOVA two-way test).

Unexpectedly, the aforementioned effect of MuSK plasma on muscle contraction appeared not to be directly related to an effect on AChRs and/or ACh release as shown in two reference models of defective neuromuscular transmission where the level of AChRs or the level of ACh release was moderately affected, namely in the α-BuTX-induced MG model with a decreased number of AChRs[28] and the Rn/Rn mutation with a decreased number of active presynaptic calcium channels.[34] In these models, both low calcium and curare caused a very marked reduction in contraction relative to controls (P. C. Molenaar, unpublished observations).

Because MuSK plasma had an effect on nerve stimulus-induced muscle contraction under the condition of low [Ca^{2+}] in the medium and not in the presence of 2 mmol/L Ca^{2+} in combination with 400 nmol/L tubocurarine, the effect of tubocurarine was reinvestigated but now in medium with low [Ca^{2+}]. For this purpose, a concentration of 0.5 mmol/L Ca^{2+} was chosen because in the presence of 0.25 or 0.35 mmol/L Ca^{2+} the contractions had become too small to evaluate quantitatively any additional effect of tubocurarine. Figure 2, upper panel, shows that in normal FDB muscles, the effect of tubocurarine was, relative to controls, increased in muscles derived from mice treated with MuSK plasma ($P < 0.05$). In notexin-treated muscles (Figure 2, lower panel), MuSK plasma showed a similar trend, but this was not statistically significant ($P = 0.09$).

The number of AChRs was measured by *in vitro* binding of ^{125}I-α-BuTX (Table 3). It can be seen that 14 days after notexin treatment the FDB muscle had bound the same amount of radiolabel as control muscles. On the other hand, at 8 days there was approximately 30% more binding in the regenerated muscle, which may be due to the fact that the endplates were larger at this time of regeneration (see below), in contrast to the situation at 14 days after notexin when this effect had subsided. Injection of MuSK plasma for 10 days in normal muscle had no effect on ^{125}I-α-BuTX binding. Similarly after notexin, the treatment with MuSK plasma for 1 to 14 days and 1 to 21 days did not cause a change in the binding of ^{125}I-α-BuTX.

Figure 3 shows that MuSK plasma had no effect on the fast turnover of newly formed AChRs after notexin injection; the half-life was 1.7 and 1.6 days, for patient and control plasma, respectively. When ^{125}I-α-BuTX was injected at 14 days, corresponding to the condition of Figure 1 and Table 2, these values had increased to 4.4 and 4.2 days for MuSK and control plasma, respectively.

Quantitative histochemistry showed that endplates were enlarged after notexin treatment compared with normal endplates (Figure 4, A–D). Moreover, in notexin-treated muscles the levels of AChRs were increased relative to

Table 3. Measurement of AChRs in Regenerating and Normal FDB Muscles

Time after toxin or PBS injection	Binding of ^{125}I- α-BuTX (cpm)		Notexin/control
	Notexin	Control	
8 days	8900 ± 440 (4)	7100 ± 350 (4)	1.30 ± 0.08*
14 days	7700 ± 570 (4)	7400 ± 510 (4)	1.00 ± 0.02
	MuSK patient 1 plasma, day 1–10	Control patient plasma, day 1–10	MuSK/Control
No notexin	6100 ± 240 (4)	5300 ± 250 (4)	1.10 ± 0.07 (4)
	MuSK patient 1 plasma, day 1–14	Control patient plasma, day 1–14	MuSK/Control
14 days after notexin	1500 ± 90 (10)	1500 ± 50 (10)	1.00 ± 0.07 (10)
	MuSK patient 1 plasma, day 1–21	Control patient plasma, day 1–21	MuSK/Control
21 days after notexin	6300 ± 180 (4)	6300 ± 260 (4)	1.00 ± 0.04 (4)

Muscles were incubated for 2 hours in the presence of 1 μg/ml ^{125}I- α-BuTX. Values of radioactivity have not been corrected for the specific radioactivity of the radiolabel used, so the values of the binding between different experiments cannot be compared.

Means ± SEM with number of muscles between parentheses. *$P < 0.05$, Student's *t*-test.

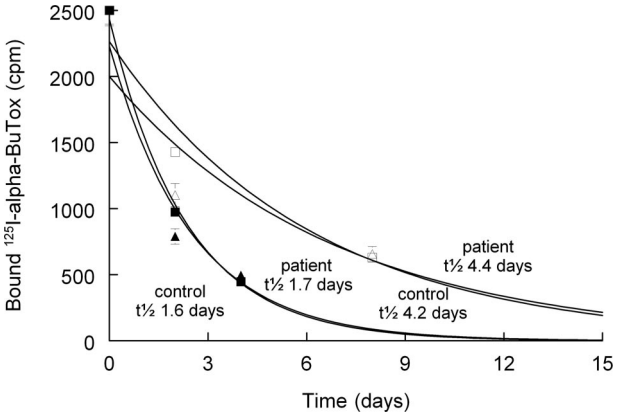

Figure 3. Loss of bound ^{125}I-α-BuTX from notexin-treated FDB muscle treated with plasma from patient 1 (triangles) and control plasma (squares). Injections of ^{125}I-α-BuTX were given (at $t = 0$ in the figure) either at day 7 or at day 13 (open symbols) after notexin-treatment. The continuous lines were exponentially fitted to the data points, with the values of half-life time as indicated in the figure. Values at $t = 0$ were normalized to 2500 cpm. Means ± SEM of four to eight muscles.

presynaptic VAChT levels (Figure 4E). Figure 4D also shows that this increased ratio of the AChR versus VAChT staining by notexin was prevented by injection of MuSK plasma, thereby reducing the AChR/VAChT ratio to the value observed in normal FDB muscles. Similarly, the notexin-induced increase of synapse area was prevented by MuSK plasma (Figure 4F). No AChR clusters were observed outside the areas that had stained for VAChT. Similar results were obtained when the endplates were stained for rapsyn; the rapsyn/VACHT ratio was reduced by MuSK plasma in notexin-treated muscles (Supplemental Figure S2, see *http://ajp.amjpathol.org*).

Discussion

The main outcome of the present investigation using the notexin model of regenerating synapses is that MuSK-MG patient's plasma causes a reduction in endplate size, whereas minimal effect was found in normal adult muscle where no such endplate regeneration takes place. Second, MuSK plasma reduces the efficacy of neuromuscular transmission under conditions of low external calcium concentration, but this effect is no greater than the effect of the MuSK-plasma in normal muscle. It will be discussed below whether or not these two effects of MuSK-MG plasma are two sides of the same coin or really different effects. Third, the magnitude of the effects of MuSK antibody on regenerating synapses proves to be rather moderate, in contrast to our prediction that the results would be relatively pronounced during the formation of synaptic contacts. Two possible explanations are that the bound antibody leaves the MuSK to exert still some of its trophic influence on synapse formation or that not enough molecules of MuSK have antibody bound to block endplate formation. It has been reported that endocytosis of MuSK is required for its physiological function,[35] and it is quite possible that binding of MuSK antibody blocks this process.

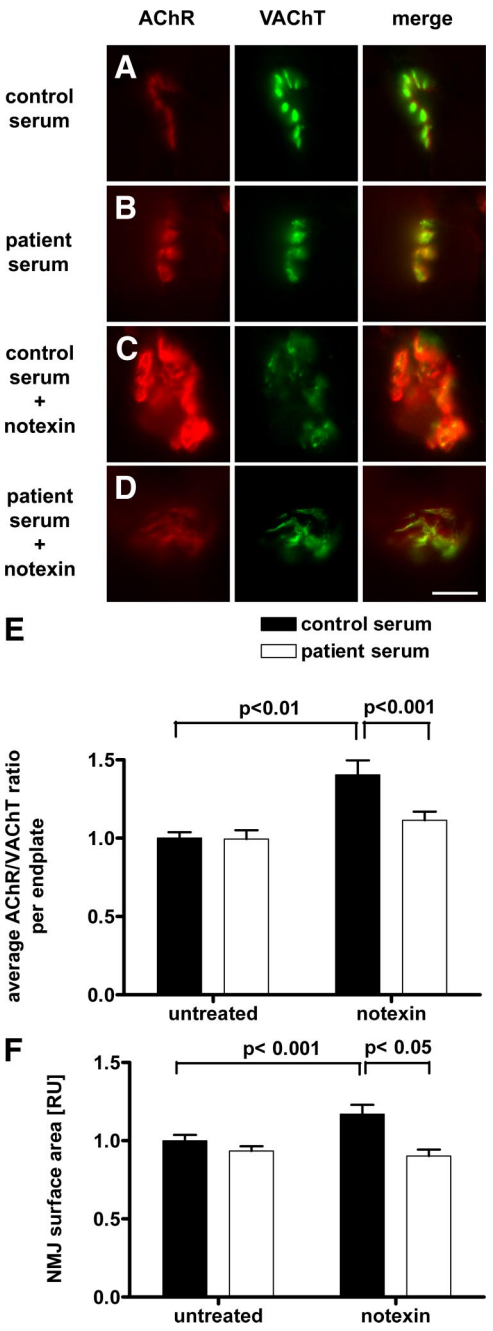

Figure 4. Immunohistochemical analysis of the NMJ using fluorescent microscopy. Muscles were analyzed after 7 days' treatment with patient's or control plasma. AChR (postsynaptic membrane) is stained in red, VAChT (nerve bouton) is stained in green; merge on the right. Scale bar is 10 μm. **A:** Normal endplate in a muscle treated with control serum. **B:** Endplate in a muscle treated with patient 1 serum appears normal. **C:** In a muscle treated with control serum, new endplates are formed 7 days after notexin treatment. These endplates are enlarged compared with normal adult endplates. **D:** Endplate in a muscle treated with patient 1 serum 7 days after notexin treatment. The enlargement of newly formed endplates is not present in this condition. **E:** Measurement of fluorescence intensities of AChR relative to VAChT staining in an average of 105 endplates per muscle. The average AChR/VAChT staining was significantly decreased at endplates of patient serum treated animals compared with control serum treated muscles in the group of 7 days after notexin. **F:** Surface area of photographed endplates of identically treated muscles. The average surface area was significantly decreased at endplates of patient serum treated animals compared with control serum treated muscles in 7 days after notexin treatment. Means ± SEM of four to six muscles. The level of statistical significance (unpaired *t*-test) is as indicated in the figure.

Notexin Model of Regenerating Synapses

The time course of degeneration and regeneration in the FDB muscle is similar to results obtained earlier by others in the soleus muscle of the rat by application of the myotoxic agents bupivacaine and notexin.[25,32] After 7 to 8 days, the regenerated muscle contracted, unfortunately, still too weakly for reliable recordings to be made so that this early time point of regeneration could not be compared with the quantitative histochemical data of Figure 4 that were obtained on day 7.

In the present experiments, the amount of ^{125}I-α-BuTX binding at 8 days after notexin injection was approximately 1.3 times higher than in controls, whereas at 7 days after notexin the histochemical staining of AChRs and rapsyn, relative to controls, was 1.5 times higher. On the other hand, at 14 days after notexin the ^{125}I-α-BuTX binding was the same as in controls not treated with notexin. This suggests that the size of the endplates had, at 7 days after notexin, transiently increased during regeneration of the fibers. This is compatible with the results by Grubb and Harris[25] who found that after an initial increase at 7 days the amplitude of MEPPs and the output of ACh had become normal again at 14 days; although, the effect on the amplitude of the MEPPs could be largely attributed to changes in the impendance of the membrane.[25]

The finding that the sensitivity to tubocurarine in notexin-treated, 14-days-regenerated FDB muscles was comparable with that of normal FDB muscle indicates that the neuromuscular transmission was as efficient as in control muscles. However, not all was "normal" in regenerated FDB muscle. First, the turnover of AChRs at 6 days was remarkably fast ($t_{1/2}$ = 2 days), and at 14 days, the turnover, although slowed down, was still two times faster than in normal muscles ($t_{1/2}$ = 5 to 6 days). Second, the regenerated fibers were rather twitchy and sometimes relaxed more slowly than controls after a stimulus. Although this was not investigated further, the picture that emerged is that regenerated muscle contained embryonic-like AChRs[36] and embryonic rSkM2 sodium channels instead of the normal sodium channels being responsible for the muscle action potential.[37] Shyng and Salpeter[38,39] have demonstrated that in denervated muscle the AChRs have a very fast turnover (approximately 1 day) when they are recently inserted at the endplate, whereas on reinnervation, a stabilization of the AChRs takes place to a half-life time of approximately 14 days. Although denervated muscle contains extrajunctional AChRs with a rapid turnover and with the gamma instead of the epsilon subunit, it is uncertain as to whether the endplate AChRs with a rapid turnover in the present regeneration experiments also had receptors with the gamma subunit and whether this was replaced gradually by epsilon subunit containing AChRs when the muscle regenerated further in 14 to 20 days.

It should be borne in mind that in regenerated FDB muscle there is not really a *de novo* formation of synapses like in embryonic muscle because the basal lamina probably largely survives the notexin treatment, so that regeneration of junctions is much faster than would be possible in the absence of extracellular matrix specialization at the site of the old endplate.

Effect of MuSK-MG Plasma on Endplates

Two main findings of this study were the effects of MuSK-MG plasma on the size of endplates and on the muscle contraction at low [Ca^{2+}]. The effect on endplate size was found only in regenerating muscle, whereas the effect on contraction was found in regenerating as well as in normal muscles. It is likely that the effect on endplate size is because of a direct, negative effect of antibody on the functioning of MuSK protein, such as the interaction between lrp4 and MuSK.[21] Whether this effect is of the same type as the fragmentation of endplates caused by the human MuSK mutation studied by Chevessier et al[40] remains to be seen; at any rate, it is a much more modest effect. These effects on morphology and contraction may be due to different mechanisms that are as yet unclear. Although the number and density of AChRs at the endplate was not influenced by the MuSK-MG plasma injections at 14 days, it is possible that the function of one of the channel properties of the AChRs was diminished by the combination of lowering [Ca^{2+}] and the MuSK-antibody interaction. An alternative possibility is that there was a presynaptic effect of MuSK antibody, eg, on terminal size. However, it is difficult to see how this can be reconciled with the results of the experiments with tubocurarine in the presence of 2 mmol/L Ca^{2+}; because under this condition, it would be expected that there is less ACh release from nerve terminals because of their subnormal size, and thus, the contraction of the muscle would have been more sensitive to tubocurarine than controls. In fact this was not the case. It is unlikely, therefore, that the increased sensitivity in low [Ca^{2+}] is simply due to a morphological effect of MuSK antibody treatment, ie, a decreased size of the synapse. In other words, because of the lack of the effect of tubocurarine at 2 mmol/L Ca^{2+}, it is likely that in the MuSK-plasma-treated muscle the safety factor of neuromuscular transmission was not affected at all at least at 14 days' regeneration of the muscle, in contrast to the situation at 7 days' regeneration when the endplace size seemed to be reduced as shown in the experiments with the histochemical staining of the AChR. However, in low [Ca^{2+}] the safety factor of neuromuscular transmission was affected more than in control muscles, possibly for reasons as outlined above, and tubocurarine compromised the safety factor then even further.

The turnover of AChRs was increased during regeneration after notexin but otherwise not influenced as a result of MuSK-MG plasma injections. It has been reported that stimulation of the MuSK-rapsyn pathway by agrin stabilizes AChRs in the endplate of denervated muscle even when the muscle is not reinnervated.[24] Therefore, it was expected that MuSK-MG plasma would exercise a negative effect on the recovery of the half-life time of the AChRs, but this turned out not to be the case.

Sera from patients with seronegative MG (including in retrospect some MuSK-MG patients) have some effect on

AChRs in tissue culture or whole muscle, eg, Refs.[9] and.[41] Exposure of TE671 cells to MuSK-MG sera reduced AChR expression by approximately 20% but had no effect on AChR subunit or MuSK mRNA expression.[42] The present results with passive immunization using MuSK-MG plasma are different from those obtained by Jha et al,[43] who used the approach of active immunization with recombinant MuSK in mice, and by Cole et al,[44] who administered i.p. injections of IgG to mice in relatively large amounts (compared with total mouse IgGs). Jha and collegues[43] found weight loss, decrease of compound muscle action potentials, and a reduction of MEPP amplitudes in isolated muscles. It is possible that the differences with the present results have been due to the production of anti-MuSK immunoglobulin IgG2 in the mice with resulting complement attack to the endplate membrane near the sites where AChRs are inserted. Cole and collegues[44] found decrement in the EMG on repetitive nerve stimulation and reduced staining of AChRs in diaphragm muscle. The reason of the discrepancy between these and our results remains to be established. Cole and collegues[44] reported that C57BL/6J mice were more susceptible than FVB/NJ mice to MuSK-MG antibody, but in our experiments, C57BL/6J mice are not particularly susceptible in contraction experiments to MuSK-MG plasma (unpublished observation).

Implications for the Pathophysiology of MuSK-MG

An interesting case of a severe congenital myasthenic syndrome has been presented in which the cause was demonstrated to be a V790M mutation of the gene coding for MuSK resulting in clusters of very small endplates; further that this abnormality could be transferred within weeks to mice after transfection of the corresponding human cDNA mutant by electroporation into the tibialis anterior muscle.[40] This unequivocally shows that malfunctioning MuSK can have rapid and severe consequences in adult muscle, even though the structure of the neuromuscular junction appears to be fairly stable during life.[45,46] Silencing of MuSK in animal studies by injection and electroporation of small interfering RNAs against MuSK-mRNA causes deterioration of the structure of endplates within 6 weeks.[23] The question arises as to whether anti-MuSK antibodies from patients can affect the function of MuSK as severely as in the abovementioned mutation, even when the amount of MuSK remains constant. The question is apparently not an easy one to answer. There also seems to be a paradox in the pathophysiology; on the one hand, symptoms in MuSK-MG can be very severe while on the other hand the laboratory findings are ambiguous, with abnormalities that seem relatively innocuous, such as slightly subnormal MEPP amplitudes with normal AChR numbers.[47–49]

In summary, the present results in mice suggest the possibility that MuSK MG autoantibodies indeed influence the molecular function of MuSK, possibly by hampering the interaction between lrp4 and MuSK,[21] with the capability thereby to diminish the size of endplates and the functioning of the AChRs without reducing their number. Obviously, such effects might be much larger in patients than in the present experimental mice because antibodies would circulate at a higher concentration and for a very much longer period than could be tested in our mice.

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