ABSTRACT: Anti-GQ1b antibodies are associated with the Miller Fisher syndrome (MFS), a variant of the Guillain–Barré syndrome (GBS). In the ex vivo mouse diaphragm, anti-GQ1b–positive MFS serum induces muscle fiber twitching, a temporary dramatic increase of spontaneous quantal acetylcholine release, and transmission blockade at neuromuscular junctions (NMJs). These effects resemble those of α-latrotoxin (α-LTx) and are induced by antibody-mediated activation of complement. We developed an assay for detection of the α-LTx–like effect, using muscle fiber twitching as indicator. We tested 89 serum samples from GBS, MFS, and control subjects, and studied correlations with clinical signs, anti-ganglioside antibodies, micro-electrode physiology, and complement deposition at NMJs. Twitching was observed with 76% of the MFS and 10% of the GBS samples. It was associated with ophthalmoplegia and anti-GQ1b antibodies in patients, and with increased spontaneous acetylcholine release and C3c-deposition at mouse NMJs. This study strongly suggests that antibodies to GQ1b (with cross-reactivity to related gangliosides) are responsible for the α-LTx–like activity. The twitching assay is an efficient test for detection of this effect, and allows for screening of large numbers of samples and modifying drugs.

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DETECTION AND PREVALENCE OF α-LATROTOXIN–LIKE EFFECTS OF SERUM FROM PATIENTS WITH GUILLAIN–BARRÉ SYNDROME

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The Guillain–Barré syndrome (GBS) is an acute, postinfectious monophasic polyneuropathy leading to a heterogeneous spectrum of motor and sensory deficits. The Miller Fisher syndrome (MFS) is a variant of GBS, characterized by ophthalmoplegia, ataxia, and areflexia.[17] Antibodies to a variety of gangliosides are present in the acute phase serum in clinical subgroups of MFS/GBS patients. Ophthalmoplegia in GBS and MFS patients is strongly associated with the presence of antibodies to GQ1b,[8,9,29,31] a ganglioside that is present at high concentration in human oculomotor nerves.[7,8] The pure motor form of GBS is associated with antibodies to GM1,[15] GM1b,[30] GD1a,[13] and GalNac-GD1a.[1] Thus, the association between antibody specificity and clinical manifestations strongly suggests that these anti-ganglioside antibodies play a role in pathogenesis of the disease.

Experiments on mouse diaphragm nerve–muscle preparations have suggested that the neuromuscular junction (NMJ) might be one of the targets of anti-ganglioside antibodies.[3–6,11,21,22,27] Anti-GQ1b–positive MFS serum, purified immunoglobulin G (IgG), and human and mouse anti-GQ1b monoclonal antibodies (mAbs) induced a more than 100-fold

Abbreviations: ACh, acetylcholine; α-LTx, α-latrotoxin; CMAP, compound muscle action potential; ELISA, enzyme-linked immunosorbent assay; EMG electromyography; EPP, endplate potential; GBS, Guillain–Barré syndrome; Ig, immunoglobulin; LPS, lipopolysaccharide; mAb, monoclonal antibody; MEPP, miniature endplate potential; MFS, Miller Fisher syndrome; NMJ, neuromuscular junction; PBS, phosphate-buffered saline; TLC, thin-layer chromatography

Key words: α-latrotoxin; GQ1b; Guillain–Barré syndrome; Miller Fisher syndrome; neuromuscular junction

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increase in spontaneous quantal acetylcholine (ACh) release, measured as miniature endplate potential (MEPP) frequency, and subsequent blockade of neuromuscular transmission.[6,11,21] Superimposition of the MEPPs triggered muscle action potentials that induced twitching of muscle fibers (Fig. 1). α-Latrotoxin (α-LTx), a spider excitotoxin, induces similar effects at the NMJ, for which reason this phenomenon was termed "the α-LTx–like effect" (Fig. 1). Ultrastructural damage with similarities to that induced by α-LTx has been observed at mouse motor nerve terminals treated with MFS serum or mouse anti-GQ1b mAbs.[19] The α-LTx–like effect of serum only occurs after subsequent incubation with serum from a healthy control subject, but not with heat-inactivated or C5-deficient serum, and is associated with depositions of C3c at NMJs of preparations.[6,11,21] These findings together suggest that the α-LTx–like effect of serum from MFS patients is mediated by complement activation at endplates induced by serum antibodies.

The standard micro-electrode technique to detect the α-LTx–like effect is laborious and requires relatively large amounts of serum, micro-electrode equipment, and specialized technical skills. The micro-electrode technique is therefore less suitable for determining the α-LTx–like effect in large groups of samples and for titrating the effect, or for screening

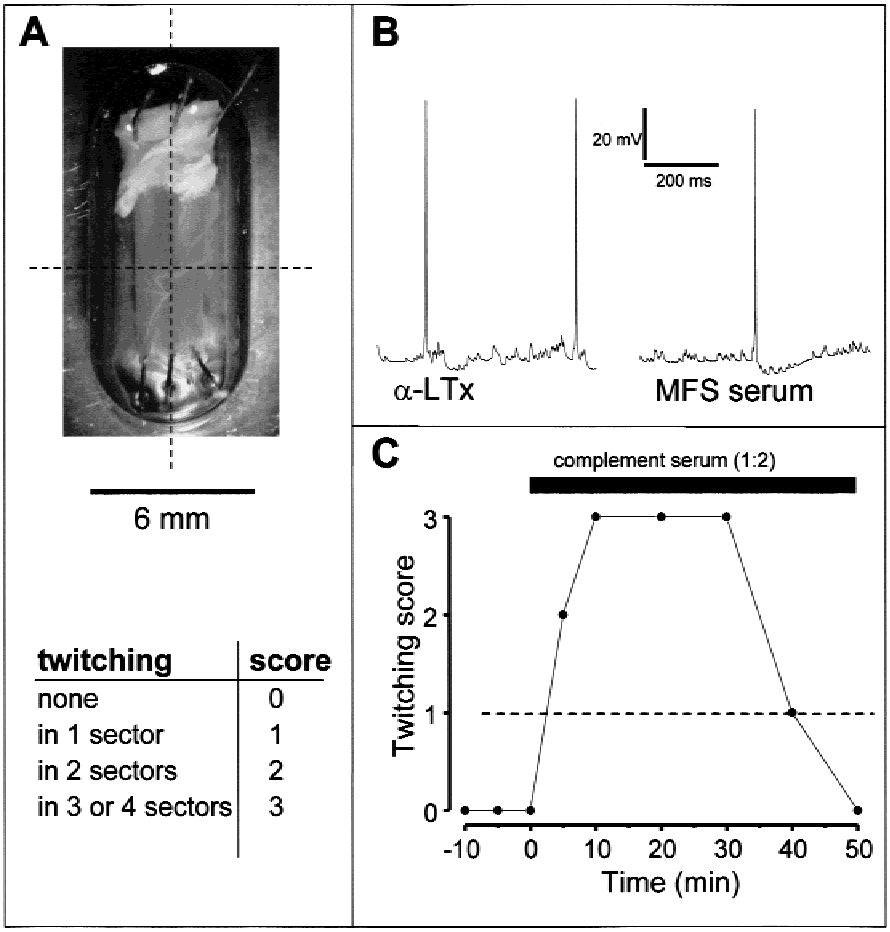

FIGURE 1. Origin and scoring of asynchronous twitching of fibers in mouse diaphragm. (**A**) A diaphragm strip pinned out in a 250 µl incubation well. The strip was divided in four imaginary sectors and the twitching grade was scored according to the table. (**B**) High-frequency miniature endplate potentials (MEPPs) trigger muscle action potentials in hemidiaphragms incubated either with 4 nM α-latrotoxin or heat-inactivated Miller Fisher syndrome (MFS) serum (1:2) with normal control serum subsequently added as complement source. Traces (600 ms) of intracellular recording in a muscle fiber near the endplate. Resting membrane potentials were −65 and −72 mV in the α-LTx and MFS serum treated fiber, respectively. High-frequency MEPPs are visible on the baseline of the trace (~60/s). Control MEPP frequency before treatment was ~0.5/s (not shown). Asynchronous muscle fiber twitches were readily observed in both preparations. (**C**) Example of the scoring of twitches in a strip that had been preincubated with the mouse monoclonal anti-GQ1b IgM antibody CGM3 (1 µg/ml). Twitching grade was scored by the observer at several time points before and after incubation of the strip with normal human control serum (1:2) as complement source. Twitch-positivity was defined as having a score >1 for at least one observation time-point.

agents which may interfere with the effect. For this reason, a limited number of samples from MFS patients and controls have been tested so far. At present, it is unknown if the α-LTx–like effect at the mouse diaphragm ex vivo can also be induced by serum from GBS patients. Moreover, the capacity of serum to induce the α-LTx–like effect and complement-deposition at NMJs in relation to the clinical manifestations, disease activity, and specificity of anti-ganglioside antibodies in patients with MFS, GBS, and controls remains to be determined.

The aim of the present study was to establish these relationships. To this end, we developed an efficient assay on the basis of the occurrence of twitching in mouse muscle ex vivo to determine the α-LTx–like effect. We applied the assay to screen for the prevalence of the effect in a large group of GBS and MFS patients and controls. The results strongly suggest that antibodies to GQ1b (with cross- or additional reactivity to other gangliosides) are responsible for the α-LTx–like activity in serum from GBS and MFS patients.

MATERIALS AND METHODS

Patient Serum Samples Used in the Screening Study. Serum samples were obtained from 17 MFS patients and 50 GBS patients. The MFS patients suffered from areflexia, ophthalmoplegia, and ataxia, and the GBS patients fulfilled the diagnostic criteria for GBS[2] and were unable to walk 10 m independently. All samples from GBS and MFS patients were obtained before treatment, and in GBS patients within 2 weeks of onset of weakness. In addition, serum samples from 5 healthy blood donors and 17 disease controls were tested. The group of disease controls included five patients with a recent infection (three patients with enteritis and culture-proven *C. jejuni* infection, one *Borrelia burgdorferi* radiculopathy, and one viral encephalitis), five patients with relapsing-remitting multiple sclerosis during relapse, five with noninflammatory polyneuropathies (three hereditary motor-sensory neuropathy and two with polyneuropathy related to diabetes mellitus), and two patients with autoimmune neuromuscular disease (one patient with myasthenia gravis and antibodies to acetylcholine receptors and one patient with Lambert-Eaton myasthenic syndrome and antibodies to voltage-gated calcium channels). Convalescence serum samples from one MFS and four GBS patients, obtained between 3 to 6 months after onset of disease, were also tested. All samples were stored at –80°C until use.

Detection of Anti-Ganglioside Antibodies in Serum. All 89 serum samples used in the screening study were tested in 1:100 dilutions in phosphate-buffered saline (PBS) with 1% bovine serum albumin for the presence of IgM and IgG antibodies to GQ1b and GM1, by enzyme-linked immunosorbent assay (ELISA) and confirmed in thin-layer chromatography (TLC) overlay, according to methods described previously.[15] All samples with anti-GQ1b antibodies or that induced twitching were also tested for IgM and IgG antibodies to GM2, GD1a, GD1b, GD3, and GT1a.

α-Latrotoxin and Monoclonal Antibodies. α-LTx (Alomone Labs, Jerusalem, Israel) and three mouse IgM mAbs (CGM3, CGM4, and CGM5) induced by immunization with lipopolysaccharides (LPS) from a *C. jejuni* isolate from a GBS patient and reacting with GQ1b, GT1a, and GD3 were used to optimize the assay.[11] In addition, we used a control mouse IgM mAb 22/18, without anti-ganglioside reactivity.[11]

Screening for Twitching. Serum samples were defrosted and incubated for 30 min at 56°C to inactivate complement. Serum samples were diluted 1:2 in Ringer medium containing (mM) NaCl 116, KCl 4.5, $MgCl_2$ 1, $CaCl_2$ 2, Na H_2PO_4 1, $NaHCO_3$ 23, and glucose 11, pH 7.4, pre-bubbled with 95% O_2/5% CO_2. Samples were dialyzed in a Slide-A-Lyzer (Pierce, Rockford, Illinois) against more than 100-fold volume of Ringer medium for at least 15 h at 8°C. Male Swiss outbred mice (3 to 4 weeks old, weighing 10 to 20 g) were killed by CO_2 inhalation, according to the Leiden University Medical Center guidelines (UDEC #00036), and their diaphragm was dissected, and placed in Ringer medium at room temperature (20–22°C). A small portion of the rib cage was left connected to the diaphragm. The two hemidiaphragms were each sectioned in four equally sized strips of ~3 mm width, by longitudinal cuttings. Each strip was pinned out on the silicone rubber-lined bottom of a 250-μl incubation well in Ringer medium (Fig. 1A). Strips were incubated in duplicate with complement-inactivated serum samples (200 μl diluted 1:2 in Ringer medium) for 2 h at 32°C and additionally for 30 min at 8°C, and subsequently adjusted in Ringer medium to room temperature for 15 min. Strips not showing sustained synchronous muscle contraction upon direct supramaximal electrical stimulation (40 Hz) with needle electrodes were excluded from the test. Subsequently, the strips were incubated at room temperature with serum from a healthy control, as a comple-

ment source, also diluted 1:2 and dialyzed in Ringer medium for at least 15 h at 8°C.

Each strip was observed at 40× magnitude using an SZ40 stereomicroscope (Olympus, Hamburg, Germany) to determine the grade of twitching at 5, 10, 20, 30, and 40 min after application of the complement source. The twitching was defined as a fast repetitive asynchronous twitching of muscle fibers (1–5 Hz). Four sectors were discriminated using the horizontal and vertical middle of the strip to grade the twitching as 0 (no contraction), 1 (twitching in one sector), 2 (twitching in two sectors), and 3 (twitching in three or four sectors) (Fig. 1A). After 40 min of incubation, the strips were rinsed with Ringer medium for 15 min, snap frozen on dry ice, and stored at –80°C for immunohistochemical studies. The serum samples were tested in random order and blinded for the diagnosis, electrophysiological results, and presence of anti-ganglioside antibodies.

Ex Vivo Electrophysiological Studies. Within left and right hemidiaphragm preparations, muscle fibers were impaled near the NMJ, with a 10–20 MΩ glass micro-electrode filled with 3 M KCl. Intracellular recordings of MEPPs were made at 20–22°C using standard recording equipment.[6] Signals were digitized and further analyzed off-line. To monitor neuromuscular transmission, the phrenic nerve was stimulated supramaximally from time to time and the resulting contraction of the muscle was judged visually.

Forty-one selected serum samples were diluted 1:2 and dialyzed with Ringer. Left hemidiaphragms were pinned out on a piece of silicone rubber and incubated in a closed vial in 1.5 ml of serum (1:2) at 32°C for 2 to 2.5 h followed by incubation at 8°C for 45 min. After incubation, the preparation was placed in Ringer medium in a 2-ml volume recording bath, re-warmed to 20°C, and at least 30 MEPPs were measured at 3–7 NMJs during a 20-min recording period. Subsequently, the Ringer was replaced with normal human serum as a source of complement (diluted 1:2 and dialyzed with Ringer) and MEPPs were measured at 7–15 NMJs during a 45-min recording period at 20°C. Control MEPPs were measured at 5–15 NMJs in the matching right hemidiaphragm in Ringer medium at 20°C without any serum preincubation.

Immunostaining. The strips were defrosted and fixed with 2% formaldehyde in PBS for 2 h at 4°C and prepared for whole mount staining in vitro as described previously.[11,21] Longitudinal sections of 10 μm were double stained with 1:2000 dilution (0.5 μg/ml) of Texas Red–conjugated α-bungarotoxin (Molecule Probes, Leiden, The Netherlands), a ligand for acetylcholine receptors, and with 1:200 dilution FITC-conjugated anti-C3c antibodies (Dako, Glostrup, Denmark) to detect deposition of activated human complement, in PBS-0.1% Triton TX for 1 h at 4°C. The slides were rinsed four times in 4°C PBS before being mounted in Citifluor antifade (Citifluor Products, Canterbury, UK) and stored at –20°C until use.

Imaging and Quantification of Immunostaining. Images were obtained by means of a Sony color CCD camera (Berlin, Germany) mounted onto a Zeiss Axioplan fluorescence microscope (Welwyn Garden City, Herts, UK) (magnification 100×) and linked to an image archiving system (Sirrius VI, Optivision, Ossett, UK).[27,28] Bitmap processing and annotation were conducted on Photo Magic and Windows Draw (both by Micrographx, Richardson, Texas). Image-analysis measurements were made using Aequitas IA image analysis software (Dynamic Data Links Ltd, Cambridge, UK). The C3c-deposition, as indicated by the FITC signal, was determined in the area of an endplate, defined by the Texas Red signal. Positivity of endplates for C3c staining was based on predetermined camera settings, and in each coded specimen at least 10 endplates were measured.

Statistical Analysis. Difference in proportions were tested with the Chi-square test without continuity correction or Fisher's exact test, and differences in medians with the Wilcoxon-Mann–Whitney *U* test. A *P*-value <0.05 was considered to be significant.

RESULTS

Optimization of Muscle Fiber Twitching Assay. The assay was first standardized using α-LTx. Incubation of 16 strips from two mouse diaphragms with 5 nM α-LTx induced twitching grade 3 in all strips within 10 min and lasted for at least 80 min. The lowest concentration of α-LTx that induced reproducible twitching was 1.25 nM.

The assay was further optimized using three mouse anti-GQ1b IgM mAbs that have been shown to induce the α-LTx–like effect.[11] Highest grades of twitching and reproducibility were achieved after incubating the strips with 25 μg/ml of mAbs for at least 2 h at 32°C. Twitching usually occurred within 5 min after adding the normal control serum and lasted for at least 20 to 40 min (Fig. 1C). The lowest concentration to induce reproducible twitching with CGM3, CGM4, and CGM5 was 0.4, 2.5, and 0.6 μg/ml, re-

spectively. The control mAb 22/18 did not induce twitching in concentrations tested up to 40 μg/ml.

Serum from patient MFS1 with anti-GQ1b antibodies and known to induce the α-LTx–like effect in the mouse hemidiaphragm preparation,[21] induced reproducible twitching only after a further incubation of 30 min at 8°C, presumably because antiganglioside antibody/antigen binding is facilitated at low temperature. Therefore, a subsequent cooling period was included of 30 min at 8°C. Serum samples from MFS1 were tested in duplicate 14 times on different days and each strip reached a twitching score of 2 (14%) or 3 (86%) within 5 to 10 min. The twitching score stayed 2 or 3 for at least 10 to 40 min. Serum from four other anti-GQ1b positive MFS patients also induced a twitching score 2 or 3 within 10 min. Serum from five healthy controls induced no twitching (grade 0) or occasionally grade 1, but never reached grade 2 or 3. Based on these findings, a serum sample with a maximum twitching score of 2 or 3 in at least one of the two duplicate strips within 30 min of observation was considered to be positive for the α-LTx–like effect.

Interobserver Variation. Samples from 33 patients (19 MFS/GBS and 14 controls) were tested in random order according to the optimized protocol, and were visually scored independently by two observers (B.C.J. and J.J.P.), using the previous defined twitching score and criterion for the α-LTx–like effect, blinded for the origin of the samples. The samples were tested in duplicate in series of four or eight sera, including MFS1 as a positive control in each series. The inter-strip agreement was 91% (kappa 0.79) and the interobserver agreement 88% (kappa 0.75).

Screening Study with Patient Serum Samples. Sera from 89 patients were screened for capability to induce twitching in the optimized assay. The sera were tested in duplicate in 14 series of 4 or 8 samples, each series including a serum from MFS1 as a positive control. The results of the screening study are given in Table 1. Twitching was only induced by sera from patients with MFS or GBS and not by controls, and was highly associated with the presence of ophthalmoplegia and serum anti-GQ1b antibodies ($P < 0.001$). Associations between twitching, microelectrode physiology, ophthalmoplegia, antibody titer, and reactivity to other gangliosides are given in Table 2.

One sample without IgM and IgG antibodies to GQ1b, GM1, GM2, GD1a, GD1b, GD3, or GT1a, however, also induced twitching and an increase in MEPP frequency. This sample was obtained from a 17-year-old male GBS patient with tetraparesis and areflexia, without sensory symptoms or involvement of cranial nerves. The first neurological symptoms developed 10 days after a flu-like syndrome with sore throat and with positive serology for Epstein–Barr virus infection. The patient was treated with intravenous immunoglobulins and made a complete recovery within 98 days.

Convalescent samples from five GBS/MFS patients, whose pretreatment samples all induced twitching, were all negative in the twitching assay (results not shown).

Association between Twitching and the α-LTx–Like Effect in Electrophysiology. Serum samples from 41 patients (17 MFS, 10 GBS, and 14 controls) were used to determine the relation between twitching and the α-LTx–like effect in standard microelectrode physiology. The MEPP-frequencies before and after incubation with heated serum were of similar magnitude, i.e., 0.38 ± 0.02/s and 0.38 ± 0.03/s in the GBS/MFS group and 0.39 ± 0.04 and 0.35 ± 0.03 in the control group. After the addition of normal serum as complement source, MEPP frequencies increased in 14 of 18 (78%) of preparations that had

Table 1. Twitching in mouse diaphragm strips in relation to diagnosis and serum anti-GQ1b antibodies of 89 serum samples from patients with Miller Fisher syndrome or Guillain–Barré syndrome and control subjects.

Diagnosis	Ophthalmoplegia	Anti-GQ1b antibodies	No. of serum samples	No. twitching (%)
Miller Fisher syndrome			17	13 (76%)
	+	+	16	13 (81%)
	+	–	1	0
Guillain–Barré syndrome			50	5 (10%)
	+	+	5	4 (80%)
	+	–	1	0
	–	–	44	1 (2%)
Other neurological diseases	–	–	12	0
Infectious controls	–	–	5	0
Normal controls	–	–	5	0

+, present; –, absent (for anti-GQ1b antibodies: titer < 1:100).

Table 2. Relation between diagnosis, ophthalmoplegia, twitching, micro-electrode physiology, C3c deposition at endplates, anti-GQ1b titer, and antibody reactivity to other gangliosides in patients with twitching- or anti-GQ1b–positive samples.

Group	Diagnosis	Ophthal-moplegia	C3c	Titer anti-GQ1b Abs		IgG or IgM Abs to					
				IgG	IgM	GM1	GM2	GD1a	GD1b	GD3	GT1a
Twitch+, ↑fMEPP+, anti-GQ1b Abs+	MFS	+	+	960	–	–	–	–	–	–	G
	MFS	+	+	13,500	–	–	–	G	–	G	G
	MFS	+	+	3,200	100	–	–	–	–	–	G/M
	MFS	+	–	400	–	–	–	–	–	–	G/M
	MFS	+	+	400	–	–	–	G	–	–	G
	MFS	+	+	200	100	–	–	–	–	–	G
	MFS	+	+	4,800	–	–	–	–	–	–	G
	MFS	+	+	800	–	–	–	–	G	G	G
	MFS	+	+	51,200	100	–	–	–	–	–	G/M
	MFS	+	+	1,600	–	–	–	–	–	–	G
	GBS	+	+	6,400	1,600	–	–	G	–	G	G/M
	GBS	+	+	12,800	–	–	–	–	–	G	G/M
	GBS	+	+	102,400	–	–	–	–	–	G	G
Twitch+, ↑fMEPP–, anti-GQ1b Abs+	MFS	+	+	8,500	500	–	–	–	–	–	nd
	MFS	+	+	1,800	–	–	–	–	–	–	nd
	MFS	+	+	20,000	–	–	–	–	–	–	nd
	GBS	+	+	1,600	400	–	–	–	–	–	G/M
Twitch–, ↑fMEPP–, anti-GQ1b Abs+	MFS	+	–	620	–	–	–	G/M	–	–	nd
	MFS	+	–	200	–	–	–	G	G	–	G/M
	MFS	+	–	400	–	–	–	–	G	–	G
	GBS	+	–	800	–	–	–	–	G	G	G
Twitch+, ↑fMEPP+, anti-GQ1b Abs–	GBS	–	+	–	–	–	–	–	–	–	–

C3c, increased percentage of endplates positive for staining with anti-C3c (>27%); Abs, antibodies; ↑fMEPP, increased frequency of miniature endplate potentials (>1.8 s^{-1}); MFS, Miller Fisher syndrome; GBS, Guillain–Barré syndrome; G, IgG; M, IgM; +, present; –, absent (in Abs: titer < 1:100); nd, not done.

been treated with serum from the GBS/MFS group and that had been twitch-positive. No increase in MEPP frequency was observed in the control group (Fig. 2). Serum samples inducing an increase in MEPP frequency above 1.8/s, i.e., the 97.5 percentile in the group of 14 controls after complement addition, were considered to be positive for the α-LTx–like effect. We found an agreement of 90% between the presence of twitching and increased MEPP-frequency (kappa 0.80). All samples negative in the twitching assay were also negative for the α-LTx–like effect when tested for with standard micro-electrode techniques. Four samples induced twitching but no increase in MEPP-frequency in the endplates sampled. They were derived from three GBS patients and one MFS patient, and all contained anti-GQ1b antibodies (Fig. 2, Table 2).

Association between Twitching and Complement-Deposition at Endplates. All diaphragm strips from the screening study were double immunostained with labeled anti-C3c antibodies and α-BTx to determine the presence of complement activation marker C3c at endplates. In the strips incubated with serum from the 22 normal and disease controls, anti-C3c staining was found only in a low percentage of endplates (4% median, 0–27%, 2.5–97.5 percentile).

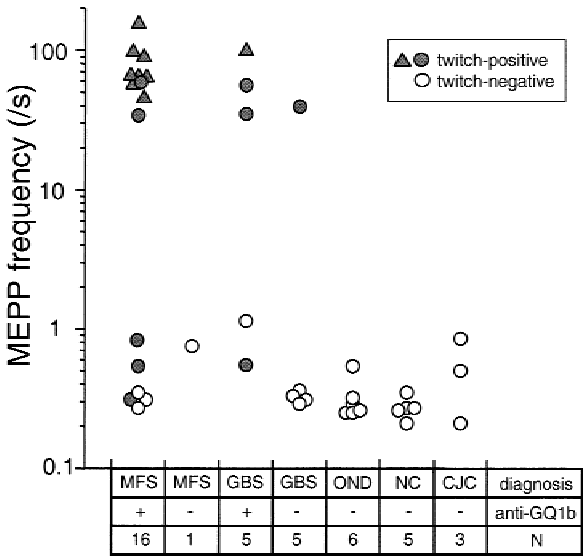

FIGURE 2. Twitching and high miniature endplate potential (MEPP) frequency as indicators for the α-latrotoxin–like effect. Forty-one serum samples from different diagnostic groups were assayed. For nine samples (triangles), the MEPP frequency data were taken from our previous study.[21] Filled triangles and circles: twitch-positive serum; open circles: twitch-negative serum. Diagnostic groups: MFS, Miller Fisher syndrome; GBS, Guillain–Barré syndrome; OND, other neurological disease; NC, normal control; CJC, *Campylobacter jejuni* infection control. +, anti-GQ1b positive; –, anti-GQ1b-negative (titer < 1:100).

The percentage of anti-C3c positive endplates was significantly higher in the 18 twitching strips (60% median, 9–82%, 2.5–97.5 percentile) than in the 71 nontwitching strips (0% median, 0–44%, 2.5–97.5 percentile, $P < 0.001$; Fig. 3). The presence of a percentage higher than 27% of anti-C3c positive endplates, i.e., the 97.5% percentile in the 22 controls, was considered to be a significant level of complement activation. This level was found in 17 of 18 (94%) twitching strips but only in 3 of 71 (4%) of nontwitching strips ($P < 0.001$). The one C3c-negative but twitch-positive sample had induced an increase in MEPP-frequency and was obtained from an MFS patient with anti-GQ1b antibodies. The three C3c-positive but twitch-negative samples were unable to induce an increase in MEPP-frequency and were obtained from two GBS and one MFS patient without anti-GQ1b antibodies. Strips incubated with convalescent samples of the five MFS/GBS patients had low percentages of C3c-positive endplates (2% median, 0–5%, 2.5–97.5 percentile).

DISCUSSION

Validity of the Muscle Fiber Twitching Assay. In this study, we developed an assay for rapid detection of the α-LTx–like activity of serum from GBS and MFS patients using twitching in mouse diaphragm strips as indicator. α-LTx induces a more than 100-fold increase in spontaneous quantal ACh release in the mouse phrenic nerve/diaphragm preparation, measured as MEPP frequency with micro-electrode techniques.[21] Anti-GQ1b mAbs and serum from MFS patients induce a similar effect via complement activation at endplates.[6,11,21] This effect is accompanied by twitching of muscle fibers at a rate of 1 to 5/s, caused by superimposed MEPPs exceeding the firing threshold of the muscle fiber. The asynchronous twitches can easily be observed by microscope at 40× magnification. The assay showed a high reproducibility in terms of inter-strip and inter- and intra-observer agreement. The sensitivity of the assay is high, as it was able to detect the effect of α-LTx in low nM range and of monoclonal antibodies in low μg/ml ranges. In 41 sera from MFS/GBS patients and controls, the sensitivity and specificity of twitching to predict high MEPP frequency in micro-electrode electrophysiology was 100% and 85%, respectively. In 89 serum samples from MFS/GBS patients and controls, the sensitivity and specificity of significant C3c-deposition at endplates was 85% and 99%, respectively. These studies show that the twitching assay reliably detects the antibody- and complement-mediated α-LTx–like effect of serum from MFS and GBS patients.

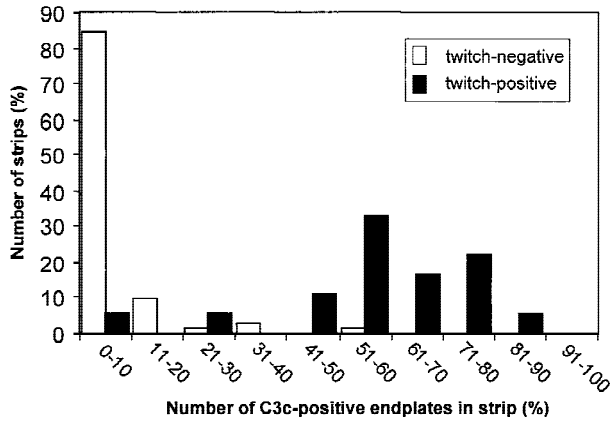

FIGURE 3. Distribution of the grade of endplate complement C3c-staining in 89 mouse diaphragm strips that were either twitch-positive (black bars) or twitch-negative (white bars) after preincubation with serum from 89 patients with Miller Fisher syndrome, Guillain–Barré syndrome, or from disease and healthy controls, with normal human control serum added as complement source. The grade of C3c staining was expressed as the percentage of endplates in a strip that were C3c-positive. A bin width of 10% was chosen. On the Y-axis, the number of strips is given as percentage of the total number of twitch-negative ($n = 71$) or twitch-positive ($n = 18$) strips in the study.

Compared to micro-electrode electrophysiology to detect the α-LTx–like effect in serum, the twitching assay has several advantages. It is rapid, requires no sophisticated electrophysiological equipment, and needs only small amounts of serum (~70 μl per strip). In addition, the assay is more sensitive in detecting the α-LTx–like effect, as with serum from 4 MFS/GBS patients with anti-GQ1b antibodies where we found twitching and C3c-deposition at endplates but failed to detect an increase of MEPP frequency at endplates sampled by micro-electrode physiology (Table 2). Moreover, higher dilutions of GQ1b-positive serum could be used in the twitching assay to detect the α-LTx–like effect. This higher sensitivity may be due to an increased permeability of the diaphragm strips at the cut edges, so that antibodies have better access to NMJs. More importantly, twitching of even a single fiber can be easily observed by microscopy, whereas with a micro-electrode and a random fiber impalement protocol, this fiber with high MEPP frequency at the NMJ would probably be missed. These advantages make the twitching assay a valuable method to investigate large groups of serum samples and monoclonal antibodies, titrate effects, conduct controlled experiments, and screen for agents to prevent or inhibit the α-LTx–like effect. This method will facilitate the research on the relation between clinical symptoms and serum antibodies and on the molecular mechanism of the α-LTx–like effect.

Prevalence of α-LTx–Like Effect and Association with Anti-Ganglioside Antibodies and Clinical Symptoms in GBS Patients. An α-LTx–like effect was previously established in serum from nine MFS patients using standard micro-electrode techniques.[21] The present study confirmed the presence of the α-LTx–like effect in serum from 13 (76%) of 17 MFS patients using the twitching assay. In addition, we demonstrated the α-LTx–like effect in acute phase serum samples from 5 (10%) of 50 GBS patients. This effect was highly associated with the presence of ophthalmoplegia and anti-GQ1b antibodies and absent in convalescent samples, providing circumstantial evidence that these antibodies induce the α-LTx–like effect. This hypothesis is further supported by the finding that human and mouse IgM monoclonal anti-GQ1b antibodies also very potently induce this effect.[11,21]

Anti-GQ1b antibodies are cross-reactive mostly with GT1a and frequently with other structurally related disialylated gangliosides such as GD1b or GD3.[24] The α-LTx–like effect could also be triggered by binding to those or other cross-reactive antigens, or by combined binding to the different gangliosides. Interestingly, the four serum samples which induced twitching but no increase in MEPP frequency in the electrophysiological studies contained relatively high titers of antibodies with reactivity to GQ1b/GT1a only. Apparently, the presence of additional reactivity to other disialylated gangliosides in sera with anti-GQ1b antibodies potentiates the ability to induce the α-LTx–like effect. The absence of twitching of anti-GQ1b/GT1a–negative GBS sera indicates that antibodies to other gangliosides are not capable of inducing the α-LTx–like effect.

Serum with anti-GQ1b antibodies from three MFS patients and one GBS patient did not induce the α-LTx–like effect, either in the twitch assay or when tested electrophysiologically. This might be related to the relatively low titer in these sera or the fine specificity of the antibodies. Alternatively, some serum anti-ganglioside antibodies have a higher affinity for gangliosides at low temperatures,[28] and may have dissociated from their target antigen in the endplate during washing or incubation with normal control serum at room temperature. Furthermore, heat inactivation, performed to remove complement, may have had a deleterious effect on antibodies in these samples. Serum from one GBS patient without ophthalmoplegia or antibodies to disialylated gangliosides (such as GD1b, GD2, GD3, GT1a, and GQ1b) also potently induced twitching and increased MEPP frequency. This interesting anomaly indicates that, in a subgroup of GBS patients, other antibodies may be able to induce the α-LTx–like effect.

Role of Complement in the α-LTx–Like Effect of Patient Serum. The induction of the twitching in our assay was strictly complement-dependent. Previous studies demonstrated that the α-LTx–like effect does not occur after incubation of nerve–muscle preparations with mouse anti-GQ1b monoclonal antibodies, heat-inactivated serum, or purified serum IgG from MFS patients alone.[6,11,21] In the present study, preincubation with the 3 monoclonal anti-GQ1b antibodies or the 89 heat-inactivated serum samples in absence of a complement source did not induce twitching or deposition of C3c at endplates (data not shown). The α-LTx–like effect was only induced after subsequent incubation with healthy control serum and deposition of complement factor C3c at endplates was highly associated with twitching. In addition, an anti-GQ1b–negative, but twitching-positive serum from a GBS patient also induced C3c depostion at endplates, whereas the four anti-GQ1b–positive but twitching-negative GBS/MFS samples did not. These findings indicate that complement-activation at the NMJs, rather than the presence of anti-GQ1b antibodies per se, is related to twitching. IgG1, IgG3, and IgM, the most frequent isotypes of anti-ganglioside antibodies in GBS and MFS patients,[14,29] are also the most potent isotypes in complement activation[10] and activate the complement cascade after binding at peripheral nerve nodes of Ranvier.[20] Hafer-Macko et al. identified complement activation marker C3d on the axolemma of motor fibers in nerve biopsies from patients with acute motor axonal neuropathy,[12] suggesting that complement activation also occurs in peripheral nerves of GBS patients.

Clinical Relevance of Muscle Fiber Twitching in GBS and MFS Patients. There are no typical clinical or electromyographic signs of muscle twitches in MFS or GBS. This may be due to the amplitudes of MEPPs and the action potential firing threshold that are known to vary between muscles and species, and may differ in NMJs from mouse diaphragm and human craniobulbar muscles. In human craniobulbar NMJs, a possible increase in MEPP frequency may not result in suprathreshold depolarizations and thereby not induce twitching. This hypothesis is supported by the finding that incubation with MFS serum of rat soleus NMJs, which have smaller MEPP amplitudes than mouse diaphragm NMJs, induces a high MEPP frequency without the concomitant occurrence of twitching (Plomp JJ, unpublished observation).

However, depletion of ACh at nerve terminals in absence of twitching may still occur in these NMJs and may block neuromuscular transmission and contribute to the craniobulbar weakness in MFS and GBS patients.

Uncini et al. reported a GBS patient with ophthalmoplegia, ataxia, areflexia, and subsequent distal tetraparesis in whom a blockade of neuromuscular transmission was suggested.[25] Serial electromyography (EMG) showed a marked reduction in distal compound muscle action potential (CMAP) amplitudes without evident changes in motor conduction velocities, distal motor latencies, or evidence for conduction blocks or morphological changes. A dramatic increase in CMAP amplitude in the following 2–3 weeks suggested that a block of ACh release from motor terminals had been present, possibly mediated by anti-GQ1b antibodies present in this patient. Interestingly, needle EMG demonstrated mild spontaneous activity in distal limb muscles. Similar EMG findings and rapid clinical recovery have also been reported in patients with the acute motor axonal neuropathy variant of GBS.[16]

Patients with MFS show motor signs similar to those of patients with botulism, in whom weakness is due to block of ACh release from motor nerve terminals by botulinum toxins.[26] Passive transfer of serum from both botulism and MFS patients induced respiratory paresis in mice.[18] Botulinum neurotoxins also bind with high affinity to GQ1b and other disialylated gangliosides.[26] The high concentration of GQ1b in human oculomotor nerves may explain the relatively vulnerability of the oculomotor muscles in botulism and MFS patients.[7,8] Also latrodectism, the disease which may develop after a bite of the black widow spider (the venom of which contains α-LTx), shows a few clinical similarities with MFS and GBS.[23] The neuromuscular junction may therefore be one of the targets in MFS and a subgroup of GBS patients.

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