Miller Fisher Anti-GQ1b Antibodies: α-Latrotoxin–Like Effects on Motor End Plates

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In the Miller Fisher syndrome (MFS) variant of the Guillain-Barré syndrome, weakness is restricted to extraocular muscles and occasionally other craniobulbar muscles. Most MFS patients have serum antibodies against ganglioside type GQ1b of which the pathophysiological relevance is unclear. We examined the in vitro effects of MFS sera, MFS IgG, and a human monoclonal anti-GQ1b IgM antibody on mouse neuromuscular junctions (NMJs). It was found that anti-GQ1b antibodies bind at NMJs where they induce massive quantal release of acetylcholine from nerve terminals and eventually block neuromuscular transmission. This effect closely resembled the effect of the paralytic neurotoxin α-latrotoxin at the mouse NMJs, implying possible involvement of α-latrotoxin receptors or associated downstream pathways. By using complement-deficient sera, the effect of anti-GQ1b antibodies on NMJs was shown to be entirely dependent on activation of complement components. However, neither classical pathway activation nor the formation of membrane attack complex was required, indicating the effects could be due to involvement of the alternative pathway and intermediate complement cascade products. Our findings strongly suggest that anti-GQ1b antibodies in conjunction with activated complement components are the principal pathophysiological mediators of motor symptoms in MFS and that the NMJ is an important site of their action.

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Guillain-Barré syndrome (GBS) is an acute, monophasic peripheral neuropathy characterized by generalized muscle weakness. In the variant of GBS termed Miller Fisher syndrome (MFS), paralysis is restricted to extraocular and occasionally other craniobulbar muscles.[1,2] Overlap cases occur in which the clinical features of GBS and MFS are intermixed. Acute phase sera from more than 90% of pure MFS and GBS overlap cases contain high titers of antibodies to GQ1b ganglioside,[3–6] which disappear during clinical recovery. Gangliosides are glycosphingolipids comprising a ceramide moiety embedded in the lipid bilayer and a sialylated oligosaccharide core exposed to the extracellular space. They are substantial components of neuronal membranes and are involved in a variety of developmental and regulatory processes.[7] In addition to reacting with GQ1b, MFS sera also react with structurally similar gangliosides including GT1a, and less frequently with GD3, GD1b, and GT1b. Both GBS and MFS are postinfectious syndromes, frequently precipitated by *Campylobacter jejuni* enteritis. Strains of *C. jejuni* isolated from MFS cases bear GQ1b-, GT1a-, and GD3-like structures on the core oligosaccharides of their lipopolysaccharides, and anti-GQ1b antibodies are thus likely to arise through molecular mimicry.[8–10]

It has been widely postulated that anti-GQ1b antibodies in MFS cause the clinical and pathological features via inflammatory demyelination of extraocular and craniobulbar nerve trunks. However, recent literature[11–13] has focused on the possibility that the neuromuscular junction (NMJ) is an important site of injury, based on the premises that (1) it lacks a blood–nerve barrier, thereby allowing antibody access; (2) cholinergic synapses are enriched in gangliosides[14]; (3)

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botulism is a major differential diagnosis for MFS and botulinum toxins bind to gangliosides present at the NMJ[15]; and (4) blockade leads to paralysis.

An earlier report described that anti-GQ1b–positive MFS sera and IgG caused a temporary and moderate increase of spontaneous quantal acetylcholine (ACh) release at NMJs of mouse hemidiaphragm preparations and concomitantly induced paralysis.[11] Other studies by Buchwald and collaborators[12,13] on the same preparation described that IgG from an anti-GQ1b–positive as well as an anti-GQ1b–negative MFS patient blocked evoked ACh release and depressed the amplitude of uniquantal postsynaptic potentials.

The goal of the present study was to prove and further study the specific role of anti-GQ1b antibodies in mediating the observed effects of MFS sera on the NMJs of mouse hemidiaphragms. In our initial experiments in which we attempted to confirm the observations of the studies referred to above, we found effects of anti-GQ1b–positive MFS whole sera and total IgG at the NMJs, which were notably different from those reported. It clearly appeared that MFS sera induced effects that were very similar to the well-known effects of the paralytic neurotoxin α-latrotoxin (αLTx)[16] at the NMJ, ie, a dramatic increase in the frequency of spontaneously released ACh quanta, without altering the amplitude of the resulting postsynaptic potentials, followed by a block of evoked ACh release resulting in paralysis. Furthermore, we found that whole MFS IgG alone was without these effects unless the preincubated muscle–nerve preparation was subsequently treated with normal serum, suggesting complement involvement.

Here we describe these novel effects of MFS sera at the mouse NMJ, and we assess the role of anti-GQ1b antibodies by studying MFS IgG subclass preparations exclusively containing the anti-GQ1b activity and a cloned human anti-GQ1b IgM from a patient with a chronic IgM paraproteinemic neuropathy resembling MFS.[17] We have also demonstrated and characterized the nature of the complement involvement, using complement-deficient sera. Our findings strongly suggest that anti-GQ1b antibodies cause the observed effects at NMJs and that they do so via intermediate components of the complement-activation cascade. Furthermore, they strengthen the hypothesis that the NMJ is an important effector site in MFS.

Materials and Methods

Patient Material

Sera were obtained from 9 patients with MFS (MFS1–9; diagnosed according to clinical criteria, including ophthalmoplegia, ataxia, and areflexia[1]) in the acute phase of the illness and from normal and disease controls. In MFS1, plasma exchange was performed. Defibrinated plasma (subsequently referred to as serum) from MFS1 and sera from MFS2–9 and controls were stored at −70°C until experimental use. From a patient Ha, suffering from a chronic IgM paraproteinemic neuropathy resembling MFS, the serum, red blood cell affinity-purified fraction (rbcM), and cloned anti-GQ1b IgM (Ha1) monoclonal antibody (mAb) has been described before.[17]

Immunological Methods and Reagents

Anti-ganglioside antibody assays were performed as described.[18] Anti-GQ1b IgG titers in the MFS sera ranged from 1:400 to 1:13,500. MFS1 and MFS2 IgG was fractionated into IgG1 + 2 and IgG3, using sequential protein G and protein A chromatography according to the manufacturer's instructions (Pharmacia, St Albans, UK) and quantitated by standard immunodiffusion techniques. All the anti-GQ1b IgG activity of MFS1 was shown to be in the IgG3 subclass; that of MFS2 was found to be in the IgG1 subclass.

Normal sera were depleted from complement C1q and C8 as described.[19,20] Completeness of C1q and C8 depletion was assessed by measuring the residual total serum hemolytic complement (CH50) in the serum by hemolysis of antibody-sensitized sheep erythrocytes (<1.0% of undepleted serum). Specificity of depletion was confirmed by showing that restoration of purified factors restored lytic activity. Also, serum from a patient with a genetic C5 deficiency[21] was used.

In Vitro Electrophysiology

Left hemidiaphragms with phrenic nerves from male Swiss mice (2–3 weeks old, 10–20 g, killed by ether) were mounted in a 2.5-ml bath with Ringer medium containing (mM) NaCl 116, KCl 4.5, $MgSO_4$ 1, $CaCl_2$ 2, NaH_2PO_4 1, $NaHCO_3$ 23, and glucose 11, pH 7.4. In some experiments, Ca^{2+} was left out and 0.2 to 1 mM EGTA was added. For oxygenation, 95% O_2/5% CO_2 was blown over the medium surface. Incubations were performed at 20°C, except for some at 32°C, as stated in the text. During incubations, fibers were impaled randomly within the preparation. Intracellular recordings of miniature end-plate potentials (MEPPs; ie, the small postsynaptic depolarizations resulting from spontaneous presynaptic release of single ACh quanta) were made at NMJs, using standard microelectrode equipment. Frequency and amplitudes of MEPPs were analyzed off-line; the mean MEPP amplitudes of end plates were corrected to a standard resting membrane potential of −75 mV. To monitor neuromuscular transmission, the phrenic nerve was stimulated supramaximally from time to time. In some experiments μ-conotoxin (2.5 μM), a specific blocker of muscle Na^+ channels,[22] was used to block muscle action potentials, to record undisturbed nerve-evoked end-plate potentials (EPPs).[23–25] Sera and antibodies were diluted with and dialyzed against Ringer before experimental use. Neuraminidase (EC 3.2.1.18, type V, from *Clostridium perfringens*) and *d*-tubocurarine were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands), αLTx from Alomone Labs, Jerusalem, Israel, and μ-conotoxin type CGIIIB from Scientific Marketing, Barnet, UK.

Immunohistology

Unfixed diaphragm tissue from MF1 mice was snap-frozen and prepared for whole mount staining in vitro as previously described.[26] Tissues were costained with α-bungarotoxin

(Molecular Probes, Leiden, The Netherlands), a ligand for acetylcholine receptors (AChRs), and anti-neurofilament antibody (Affiniti, clone 1217; Exeter, UK) to delineate the end plate and the terminal motor axon. The MFS1 IgG3 fraction (30 μg/ml) and the human mAb Ha1 (40 μg/ml) were used to localize the epitopes recognized by anti-GQ1b antibodies. Electrophysiological control experiments (data not shown) on diaphragms from 4 MF1 mice (19–32 g) demonstrated that MFS1 serum and purified IgG induced effects that were identical to those observed in muscles from Swiss mice. Immunodeposition of human antibody and complement products (anti-C3c, Dako, Glostrup, Denmark) was examined in MFS1 serum–exposed preparations from Swiss mice.

Results

Effects of MFS Sera on End-Plate Electrophysiology

Incubation of mouse diaphragm muscle–nerve preparations at 20°C with MFS sera (Table) resulted in twitching of muscle fibers at a rate of 0.1 to 5/sec, starting in superficial fibers at 10 to 30 minutes. Also, fasciculations, ie, contraction of whole motor units, were occasionally seen. Microelectrode measurements revealed an extremely high frequency of MEPPs. At many end plates it was >200/sec (Fig 1a), whereas the mean value before MFS serum was only about 0.5/sec (see Table). Sometimes, superimposed MEPPs exceeding the firing threshold triggered an action potential (not shown). This was causing the twitches, as they were prevented by block of AChRs with 2.5 μM *d*-tubocurarine, excluding an effect of MFS sera on mechanisms downstream of AChRs, eg, muscle Na^+ channels.

At some end plates, the time course of the effect could be monitored. At onset, MFS sera induced bursts of high-frequency (10–50/sec) MEPPs (see Fig 1b) of which the incidence gradually increased until the MEPP frequency became continuous (>100/sec) after 15 minutes to 1 hour. In μ-conotoxin–paralyzed preparations, EPPs could be evoked by nerve stimulation up to this point, in spite of the high MEPP frequency. However, after 15 to 30 minutes, EPPs decreased in amplitude and disappeared (Fig 2), whereas MEPPs remained highly frequent. After another 15 to 30 minutes, MEPP frequency declined and bursts reappeared until another hour later it decreased to normal values, sometimes becoming zero. Because of variable times of

Taphragm

Material	Dilution (Serum:Ringer)	MEPP Frequency (sec^{-1})		Muscle Fiber Twitching	Subsequent Incubation with Normal Serum (1:1.5)	
		Before Incubation	During Incubation		MEPP Frequency	Muscle Fiber Twitching
Sera						
MFS1[a]	1:2	0.36 ± 0.05 (4, 15, 1 hr)	123.33 ± 2.24 (4, 8–12, 30–45 min)	+	ND	ND
MFS2[a]	1:2	0.36 ± 0.03 (1, 15, 1 hr)	96.42 ± 21.10 (1, 15, 45 min)	+	ND	ND
MFS3[a]	1:2	0.45 ± 0.04 (1, 25, 1 hr)	63.33 ± 19.61 (1, 12, 1 hr)	+	ND	ND
MFS4	1:1.5	0.50 ± 0.06 (1, 14, 45 min)	44.91 ± 13.61 (1, 23, 2 hr)	+	ND	ND
MFS5	1:1.5	0.40 ± 0.06 (1, 15, 45 min)	0.87 ± 0.17 (1, 38, 2.5 hr)	−	56.49 ± 19.70 (1, 15, 1 hr)	+
MFS6	1:2	0.48 ± 0.05 (1, 15, 30 min)	65.30 ± 12.25 (1, 30, 2 hr)	+	ND	ND
MFS7	1:1.5	0.30 ± 0.06 (1, 12, 45 min)	0.47 ± 0.04 (1, 41, 2 hr)	−	98.38 ± 31.41 (1, 12, 1 hr)	+
MFS8	1:2	0.26 ± 0.03 (1, 15, 30 min)	0.40 ± 0.06 (1, 10, 1 hr)	−	88.63 ± 40.96 (1, 11, hr)	+
MFS9	1:2	0.39 ± 0.05 (1, 15, 45 min)	153.95 ± 38.00 (1, 10, 1 hr)	+	ND	ND
MFS1						
Serum (32°C)	1:1.5	0.72 ± 0.06 (2, 15, 30 min)	0.74 ± 0.26 (2, 15, 30 min)[b]	−	ND	ND
Heated serum	1:1.5	0.29 ± 0.09 (2, 12–14, 30 min)	0.28 ± 0.07 (2, 15–28, 1–2 hr)	−	113.40 ± 23.89 (2, 15, 45 min)	+
Total IgG	1.2–1.9 mg/ml	0.49 ± 0.03 (5, 5, 15 min)	0.47 ± 0.05 (5, 30–45, 2–3 hr)	−	42.48 ± 15.11 (3, 15, 20–40 min)[c]	+
Flow-through[d]	0 mg/ml	0.53 ± 0.10 (2, 15, 1 hr)	0.42 ± 0.01 (2, 45, 3 hr)	−	ND	ND
IgG1 + IgG2	1.5–2.0 mg/ml	0.37 ± 0.13 (2, 15, 1 hr)	0.45 ± 0.18 (2, 30–45, 2–3 hr)	−	0.37 ± 0.18 (2, 15, 1 hr)	−
IgG3	0.07 mg/ml	0.31 ± 0.01 (2, 15, 1 hr)	0.32 ± 0.02 (2, 30–45, 2–3 hr)	−	85.14 ± 24.46 (2, 15, 1 hr)	+
MFS2						
IgG1 + IgG2	2.1 mg/ml	0.36 ± 0.05 (1, 10, 30 min)[e]	0.51 ± 0.09 (1, 7, 15 min)[f]	−	173.33 ± 30.81 (1, 15, 45 min)	+
IgG3	0.24 mg/ml	0.55 ± 0.08 (1, 10, 30 min)[e]	0.64 ± 0.09 (1, 7, 15 min)[f]	−	0.73 ± 0.13 (1, 15, 45 min)	−

Mean ± SEM values of the number of end plates or muscles. All incubations were done at 20°C, unless indicated otherwise. Number of muscles, number of end plates per muscle, and duration of the incubation per sampling period are indicated in parentheses.

[a]Also studied earlier.[11]

[b]Subsequent 20-minute cooling period (4°C) did result in high-frequency MEPPs (207 ± 25/sec, n = 2 muscles, 5 end plates per muscle, 20-minute recording period).

[c]We attempted to facilitate microelectrode recording by blocking contractions with 1 μM tetrodotoxin, just after normal serum addition. We waited 20 minutes to allow contractions to fade, but it appeared that many end plates had already returned to lower MEPP frequencies, resulting in a relatively low mean value compared with that obtained with whole serum.

[d]IgG free fraction obtained in the IgG purification procedure.

[e]Measurements done in contralateral (right) hemidiaphragm.

[f]Measurements done in Ringer at 20°C, after a 3-hour incubation period with IgG at 32°C and 45 minutes at 4°C.

ND = not done.

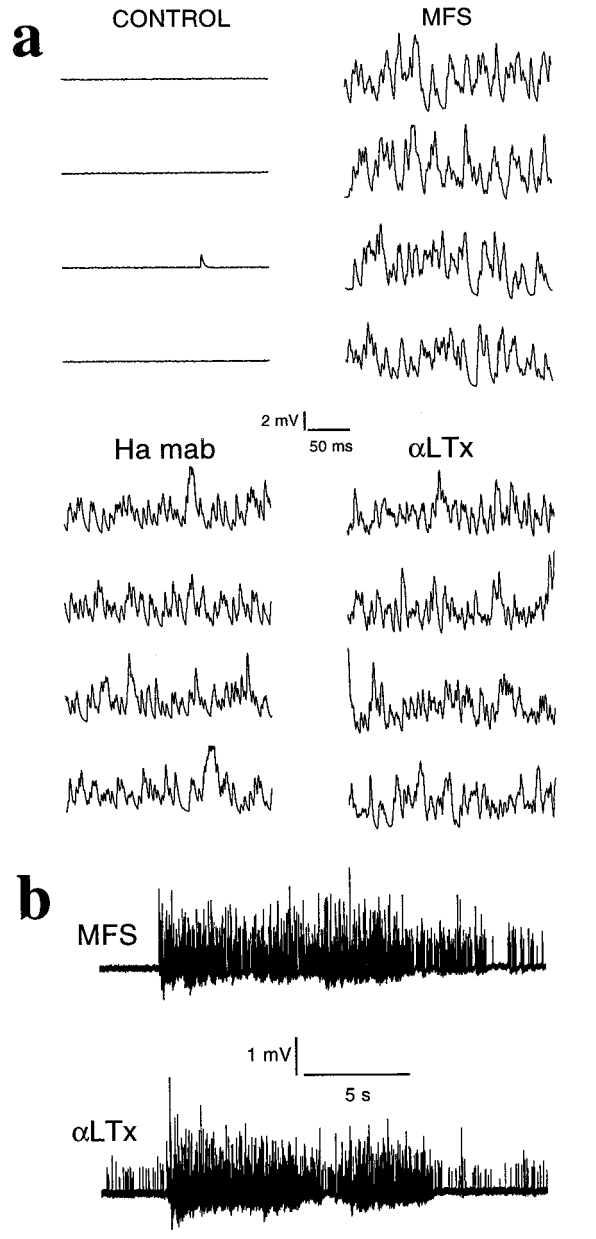


Fig 1. Examples of electrophysiological recordings of the effects of Miller Fisher syndrome (MFS) sera (1:1.5–2), the human monoclonal (mAb) Ha1 anti-GQ1b IgM (0.5 mg/ml; normal control serum added to provide complement), and 3 nM α-latrotoxin (αLTx) at mouse diaphragm neuromuscular junctions (NMJs). (a) Subsequent 250-msec traces, plotted below each other, of miniature end-plate potential (MEPP) recordings during incubation with Ringer alone, MFS serum, Ha1, or αLTx. (b) MEPP bursts at onset of the effect of MFS serum (top trace) or αLTx (bottom trace).

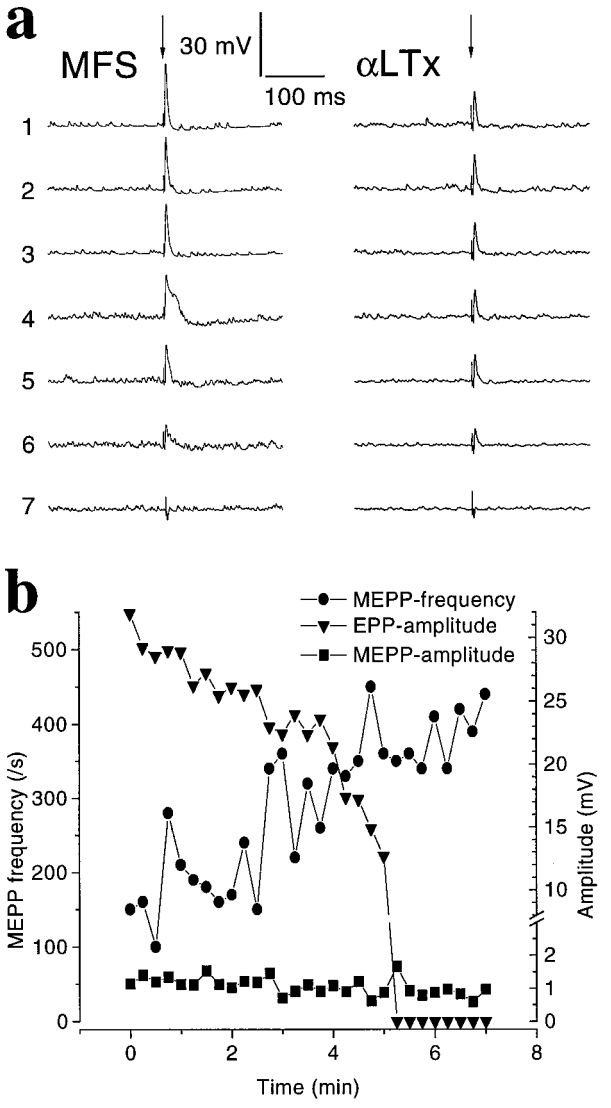


Fig 2. (a) Examples of the reduction and blocking of end-plate potentials (EPPs) during incubation with serum from 1 patient with Miller Fisher syndrome (MFS; left) or α-latrotoxin (αLTx; right). The phrenic nerve was stimulated supramaximally (arrows) *every 15 seconds; shown is each fourth trace, ie, the one recorded on every whole minute as indicated at the left side of each trace. Time point zero relates to the start of the recordings, short after the impalement of the muscle fiber at the neuromuscular junction, in the MFS example 36 minutes after the start of the incubation with MFS1 serum (serum from 1 patient with MFS). (b) Quantification of the recordings from the experiment with MFS serum depicted in a. Shown are the time courses of miniature end-plate potential (MEPP) frequency, MEPP amplitude, and EPP amplitude. Amplitudes were normalized to a standard resting membrane potential of −75 mV.*

onset throughout the preparation (presumably due to diffusion barriers for the acting factor), all stages of the effect were encountered on random impalements in a muscle at 0 to 3 hours after the incubation start. After 5 hours, however, many end plates showed no MEPPs, and on nerve stimulation large areas of the muscle did not contract.

The reversibility of the effect was studied. We incubated preparations with MFS1 serum (1:2) for 30 to

45 minutes until the effect was well under way, ie, there were many contracting fibers and end plates with high-frequency MEPPs. Then, the serum was washed away with Ringer and the preparation was monitored and fibers were randomly impaled with the microelectrode for more than an hour. It appeared that MEPPs at affected end plates remained highly frequent for more than 30 minutes (high-frequency MEPP end plates remained to be encountered for at least 50 minutes after the start of the washout) but that no new end plates became affected. Fiber twitches were observed during the washout period, but their incidence gradually lessened. Furthermore, the parts of the muscle that were paralyzed, ie, that did not contract on stimulation of the nerve, remained paralyzed during the washout period and no MEPPs could be found at end plates in those regions, indicating that slowly reversible or irreversible block of synaptic transmission had been induced by the MFS serum.

To obtain information about dose responsiveness, different serum dilutions were studied. However, it should be borne in mind that the preparation does not lend itself well to kinetic studies, due to internal (synaptic) diffusion barriers. MFS1 serum diluted at 1:5 with Ringer was still effective, although the onset of the effect was slower and the effect was less widespread within the preparation, as judged visually from the occurrence of fiber twitches. The MEPP frequency at those end plates that were affected was as high as observed with the 1:2 diluted MFS1 serum (>200 at some end plates). At a 1:10 Ringer dilution, MFS1 serum was not effective anymore during a 2-hour incubation period.

Each of the nine studied MFS sera had similar effects (see Table), although the scale, ie, number of end plates showing high-frequency MEPPs, was somewhat variable. Furthermore, the effect of some MFS sera required subsequent incubation with normal serum. This suggested that the effect was dependent on complement (see below), apparently not sufficiently present in these MFS sera, most likely due to loss of activity during storage. Control sera from subjects with a polyneuropathy without anti-GQ1b antibodies, a *Campylobacter* infection without neurological symptoms, and an influenza infection did not induce the MFS effect, even after subsequent incubation with normal serum (mean MEPP frequency before and during incubation with these sera and subsequent normal control serum ranged from 0.28 to 0.56/sec, 0.33 to 0.65/sec, and 0.25 to 0.60/sec, respectively). Sera from 4 normal control subjects were also negative. At the end of each of these control experiments, MFS1 serum was used as a positive control (not shown).

The mean amplitude of MEPPs tended to increase modestly during incubations. However, this was not statistically significant when tested for with a paired *t* test and was observed with the four normal control sera (0.98 ± 0.03 mV before and 1.15 ± 0.07 mV during incubation; mean ± SEM values), the four infection control sera (1.08 ± 0.06 mV before and 1.21 ± 0.03 mV during incubation; mean ± SEM values), and the nine MFS sera (1.17 ± 0.04 mV before and 1.27 ± 0.04 mV during the incubation; mean ± SEM values).

Resemblance with α-Latrotoxin

Incubation of mouse diaphragms with 3 nM αLTx had effects that resembled those of MFS sera, ie, (1) extremely high-frequency MEPPs (>100/sec, see Fig 1a) appearing in bursts at onset (see Fig 1b; described before by others[27,28]), (2) twitching of muscle fibers caused by superimposed MEPPs, (3) a gradual decreasing EPP that was eventually blocked, whereas MEPPs remained highly frequent for some time (see Fig 2), leading to (4) block of neuromuscular transmission. In a preparation that had been treated with an MFS serum, 3 nM αLTx failed to induce further effects (not shown).

Anti-Ganglioside IgG and Complement Involvement

At 32°C, MFS1 serum had no effects (see Table), whereas a subsequent cooling period at 4°C brought about effects as described above within a few minutes on return to 20°C. Such a temperature dependency is compatible with involvement of anti-ganglioside antibodies, as their avidity to gangliosides is in general inversely correlated with temperature.[18,29] Furthermore, MFS1 serum lacked effect on preparations that had been treated with neuraminidase (Fig 3) to remove sialic acids from gangliosides and related glycoconjugates, a procedure that by itself had no effect on MEPP frequency (0.37 ± 0.09/sec before and 0.42 ± 0.05/sec after; n = 2 muscles, 6–11 end plates per muscle; mean ± SEM values).

Purified MFS1 IgG, flow-through (the IgG-free fraction obtained in the purification process), or a mixture of both failed to induce the effects seen with whole serum, indicating that an active unstable serum factor had been lost, probably complement. Therefore, we preincubated preparations with MFS1 IgG and subsequently treated them with normal control sera to provide complement. High-frequency MEPPs and fiber twitches (see Table), such as seen with whole MFS1 serum, appeared within a few minutes. Heating MFS1 serum for 30 minutes at 56°C to destroy complement[30] abolished its effects, but subsequent addition of normal serum brought them about (see Table). To characterize complement involvement, diaphragms pretreated with MFS1 IgG were exposed to sera lacking either complement factor C1q, C5, or C8. High-frequency MEPPs and muscle fiber twitches could be evoked at the usual scale with C1q-depleted and C8-depleted sera but not with serum from a genetic C5-

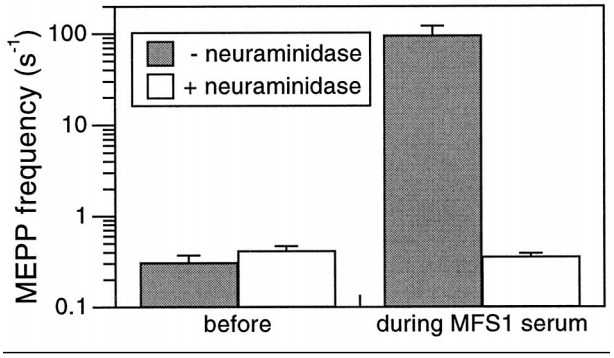
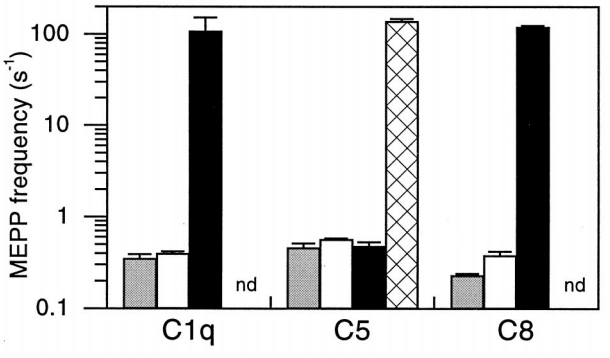


Fig 3. Effect of serum from 1 patient with Miller Fisher syndrome (MFS1; 1:2) at 20°C on miniature end-plate potential (MEPP) frequency of mouse hemidiaphragms pretreated with neuraminidase (0.6 mg/ml [2 U/ml] for 1 hour at 32°C; white columns; data are mean ± SEM values of two muscles; six to 11 end plates per incubation period) or Ringer (gray columns; n = 1 contralateral hemidiaphragm, 5 and 15 end plates sampled before and during MFS1 serum incubation).

deficient patient (Fig 4). This was not due to insufficient MFS1 IgG binding, as subsequent incubation with normal control serum readily brought about the effect. MFS1 IgG had no significant effect on the amplitude of MEPPs (mean value, normalized to −75 mV resting membrane potential, was 1.08 ± 0.08 mV before and 1.22 ± 0.07 mV during a 2–3-hour incubation; n = 5 muscles, at least 15 end plates per incubation period; mean ± SEM values).

Fig 4. Effect of C5-deficient and C1q-depleted and C8-depleted sera (1:1.5) on miniature end-plate potential (MEPP) frequency in mouse diaphragms pretreated with serum IgG from 1 patient with Miller Fisher syndrome (MFS1; 1.7 mg/ml). Preparations were subsequently incubated in Ringer (30 minutes, gray columns), MFS1 IgG (1–2 hours, white columns), and serum lacking the indicated complement factor (45 minutes, black columns). In the experiments with C5-deficient serum, normal control serum (45 minutes, cross-hatched column) was added at the end. Data are mean ± SEM values of three (C1q experiment) or two muscles (C5 and C8 experiments); 10 to 30 end plates sampled per incubation period; nd = not done.



With most MFS patients, anti-GQ1b antibodies in serum are exclusively contained in the IgG1 or IgG3 subclass.[29,31] We tested the IgG subclass fractions of MFS1 and MFS2, of which the anti-GQ1b activity was in the IgG3 and IgG1 subclass, respectively. Muscle–nerve preparations preincubated with MFS1 IgG3, but not those with IgG1 + 2, showed high-frequency MEPPs and twitches on subsequent incubation with normal control serum (see Table), as seen with those preincubated with total MFS1 IgG. The same result was obtained by using the IgG1 + 2 subclass from MFS2, whereas the IgG3 subclass was without effect.

Ca^{2+} Dependency of the Effect of MFS Sera

The action of some of the factors of the complement cascade is dependent on Ca^{2+}, whereas that of αLTx on ACh release at NMJs is not.[32] Therefore, we were interested in the dependency on Ca^{2+} of the onset and prolongation of the effect of MFS sera on neurotransmitter release. Incubation of a preparation with MFS1 serum diluted and dialyzed against 0 mM Ca^{2+}-Ringer did not result in high-frequency MEPPs. However, subsequent incubation with normal serum dialyzed against 2 mM Ca^{2+}-Ringer did result in high-frequency MEPPs (Fig 5a). When MFS1 serum containing 2 mM Ca^{2+} was replaced with 0 mM Ca^{2+}-Ringer when the effect at many NMJs was already well under way, ie, after about 20 minutes, high-frequency MEPPs remained to be found for more than 1 hour, showing that the effect, once started, is independent of Ca^{2+} (see Fig 5a and b).

Effect of a Human Anti-GQ1b Monoclonal Antibody

From a patient Ha, suffering from a chronic IgM paraproteinemic neuropathy resembling MFS, the serum, rbcM, and Ha1 mAb has been described before.[17] In view of the high molecular weight of IgM and therefore the expected difficulty in penetrating the preparation, a different protocol was used. Preparations were incubated with Ha rbcM at 32°C for 4 hours to allow penetration. Subsequently, the bath was cooled to 4°C for 45 minutes to facilitate anti-ganglioside antibody binding. Thereafter, measurements were started at 20°C. Incubation with 0.95 mg/ml rbcM did not have an effect on its own, but on replacement with normal serum (1:1.5), muscle fiber twitches and high-frequency MEPPs appeared (107 ± 32/sec, n = 5 end plates; mean ± SEM values). The same was found with 0.5 mg/ml IgM mAb Ha1 (see Fig 1a), although more end plates were affected. The mean MEPP frequency rose to 180 ± 40 (n = 10 end plates; mean ± SEM values). Control experiments with a mixture of normal human IgG (3.2 mg/ml) and IgM (0.5 mg/ml) showed that preparations remained viable during the warming/cooling/rewarming procedure (tested for with

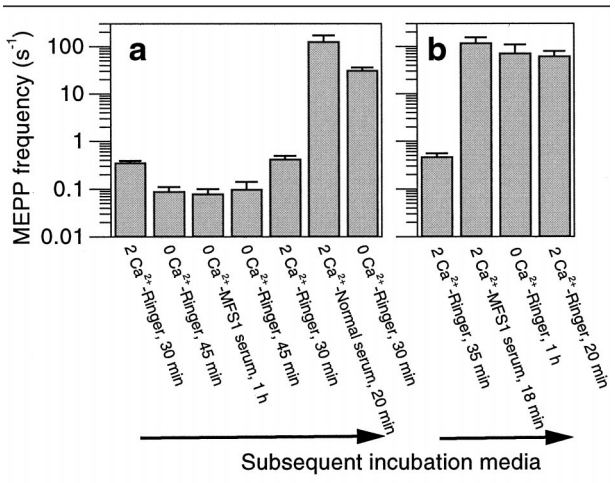


Fig 5. Ca^{2+} dependency of the effect of serum from 1 patient with Miller Fisher syndrome (MFS1; 1:2) on miniature end-plate potential (MEPP) frequency in mouse diaphragm at onset (a) and when well under way (b). The millimolar Ca^{2+} concentrations of subsequent media are indicated by x*-axis labels; 1 mM (a) or 0.2 mM (b) EGTA was added to 0 mM Ca^{2+} media to buffer any remaining Ca^{2+}; mean ± SEM values of five to 11 end plates of one muscle.*

indirectly evoked muscle contraction) and that the MEPP frequency did not change significantly (0.85 ± 0.10/sec before and 0.48 ± 0.08/sec after IgG/IgM incubation; 0.48 ± 0.03/sec during subsequent incubation with normal serum [1:1.5]; n = 2 muscles, 6–15 end plates per measuring period; mean ± SEM values).

Anti-GQ1b Antibody and Complement Deposition at End Plates

Immunohistology showed that IgM mAb Ha1 binds to several structures in the mouse diaphragm preparation, such as intramuscular capillaries and motor axons. Furthermore, binding to NMJs was shown by colocalization of staining with Ha1 and fluorescently labeled α-bungarotoxin (Fig 6a–c), confirming earlier work.[17] Also, MFS1 IgG3 bound to NMJs (see Fig 6d–f). In a preparation treated with MFS1 serum in the same way as in the electrophysiological experiments, both IgG (not shown) and complement C3c deposition was seen at NMJs (see Fig 6g–i).

Discussion

Our data demonstrate that MFS sera and serum fractions induce severe electrophysiological defects at mouse NMJs in vitro, comprising massive release of ACh quanta, followed by transmission block. Several findings indicate that anti-GQ1b ganglioside antibody is the responsible serum factor. First, all the MFS sera were positive for anti-GQ1b antibody, which is highly specific for the disease, and control sera did not have effects. Second, the effect of MFS1 serum was abolished by neuraminidase pretreatment of the muscle preparation, which would remove the relevant anti-GQ1b–binding disialosyl structures from GQ1b and related sialylated glycoconjugates. Third, the IgG3 fraction of MFS1, which contains all anti-GQ1b IgG activity and yet comprises only 7% of the total serum IgG, induced the effect, whereas the IgG1 + 2 fraction, containing more than 90% of the total serum IgG and no anti-GQ1b antibodies, had no effect. Fourth, immunohistological data confirmed binding of MFS1 IgG3 and human monoclonal anti-GQ1b IgM to mouse NMJs. Fifth, and most important, the human monoclonal IgM against GQ1b (and the closely related disialosyl gangliosides GT1b, GD1b, and GD3)[17] initiated effects at NMJs that were identical to those of MFS sera.

In addition to providing evidence for anti-GQ1b antibody involvement, our data provide proof for involvement of the complement system in the effect of MFS sera at NMJs. Because the effect is mediated by IgG, one would predict classical pathway involvement.[33] However, C1q-depleted serum still mediated the effect of MFS1 IgG, indicating that the classic pathway, comprising C1-complex formation by C1q, C1r, and C1s, is not necessarily involved. This suggests involvement of the alternative pathway, normally activated by microbial carbohydrate surfaces. One explanation might be that anti-GQ1b antibodies cross-link gangliosides or cross-reactive antigen at NMJs in such a way that carbohydrate clusters are formed that stimulate the alternative pathway. In contrast, sugar surfaces could be formed that bind mannose-binding protein, a Ca^{2+}-dependent initiator of the recently described lectin pathway.[34] This would also explain the Ca^{2+} dependency of the onset of the effect of MFS1 serum on MEPP frequency. Our finding that C5-deficient serum lacked effect after MFS1 IgG pretreatment excludes the possibility that any factor before C5 in the complement cascade acts as sole mediator.

It might be thought that the C5b-9 membrane attack complex, the pore-forming general end product of the different pathways in the complement cascade, is formed at nerve terminals, allowing Ca^{2+} flux to stimulate ACh release. This possibility, however, is excluded by the finding that C8-depleted serum retained the full electrophysiological effect. In addition, the effect of MFS1 serum on MEPP frequency, provided that it is well under way, is independent of extracellular Ca^{2+}. Furthermore, ACh release induced by nerve action potentials remained possible for some time after onset of high MEPP frequency, arguing against gross presynaptic depolarization induced by an ion leak via the membrane attack complex. Our results do not exclude an effect of an incomplete terminal complement complex C5b-7, which has been shown to stimulate second messengers at target cells such as cyclic AMP,

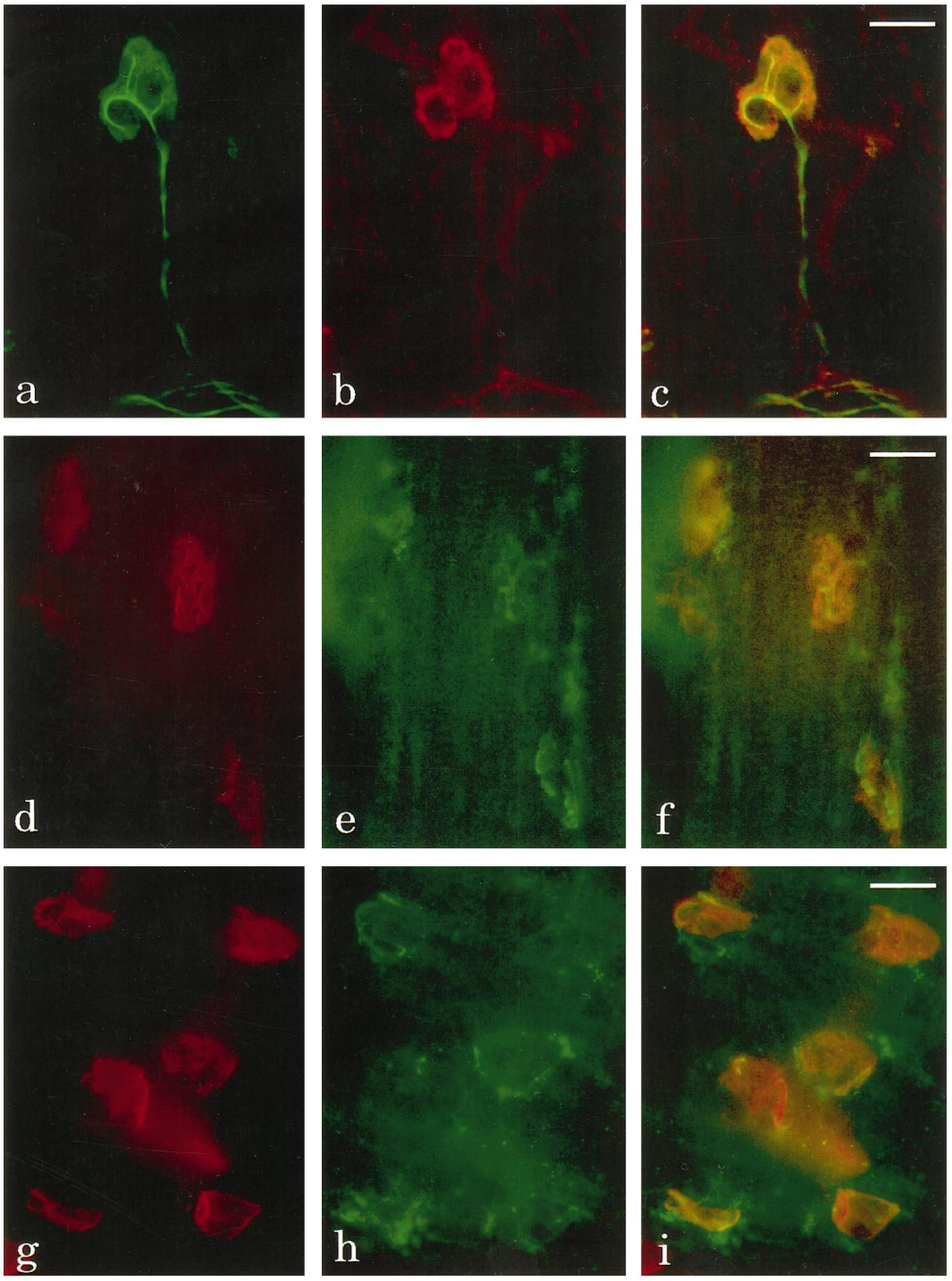


Fig 6. Immunohistology of Miller Fisher syndrome (MFS)-associated antibodies and complement at mouse neuromuscular junctions. (a–f) Whole mount mouse diaphragm stained green (a) for acetylcholine receptors (AChRs) (with α-bungarotoxin, a specific ligand) and neurofilament, showing both end-plate region and terminal motor axon, stained red (b) with human monoclonal IgM Ha1 (reactive with GQ1b, GT1b, GD1b, and GD3), and a and b overlaid in c; stained red for AChRs (d), green with IgG3 from 1 patient with MFS (MFS1; e), and d and e overlaid in f. (g–i) Localization of activated complement component C3c in MFS1 serum–treated mouse diaphragm, stained red for AChRs (g), green with anti-C3c antibody (h), and g and h overlaid in i. Scale bars = 20 μm.

inositol phosphate intermediates, and arachidonic acid metabolites[35], known stimulators of ACh release at NMJs[36], although not to such a large extent as seen here with MFS sera. In addition, our data do not preclude a role for the C5b-9 membrane attack complex in mediating other aspects of the nerve injury that may occur in MFS.

Considerable histological and immunochemical evidence supports a role for antibody-dependent complement activation in GBS.[37–40] However, complement involvement in effects of MFS sera or IgG at NMJs has not been suggested.[11–13] By using a perfused macro patch-clamp technique, Buchwald and associates[12,13] observed that at mouse NMJs, purified MFS IgG (from an anti-GQ1b–positive as well as an anti-GQ1b–negative patient) alone induces a 100- to 1,000-fold reduction of quantal content (the number of ACh quanta released per nerve impulse). Our observations differ from this in that neuromuscular transmission remained possible in nerve–muscle preparations treated with MFS IgG or complement-inactivated MFS1 serum (and also with MFS5, MFS7, and MFS8 sera before addition of normal serum), thereby excluding a gross abnormality of quantal content. Here, we only observed a reduction of quantal content sufficient to produce transmission block (as defined by block of EPPs) at NMJs already showing complement-mediated high-frequency MEPPs. That MEPPs at such NMJs persisted for some time after the block of EPPs indicates that the block was not due to depletion of ACh vesicles (or to AChR block). It remains to be seen whether the transmission block is a primary effect of anti-GQ1b antibody or is secondary to deleterious effects of massive, uncontrolled exocytosis. Another contradiction between the studies of Buchwald and associates[12,13] and our study is that a depressing action of MFS IgG on the amplitude of uniquantal postsynaptic responses was not observed by us, neither in the experiments with whole MFS sera nor in those with purified MFS IgG. The reason for the apparent discrepancies is at present unclear.

The neurotoxin αLTx, a component of black widow spider venom, is known to induce massive quantal ACh release at the NMJ via specific presynaptic receptors,[41] linked to the ACh release machinery.[42,43] The effect of MFS serum at mouse NMJs closely resembles that of αLTx in that (1) high-frequency MEPPs appear in bursts at onset; (2) once underway, it is independent of extracellular Ca^{2+}[32]; (3) nerve action potential–evoked ACh release remains possible for some time at NMJs showing high-frequency MEPPs, but it eventually fails. Therefore, it might be that αLTx receptors or downstream effector mechanisms are targeted in MFS by complement products that are activated after (cross-reactive) binding of anti-GQ1b antibodies to epitopes on, or adjacent to, αLTx receptors, one of which is a sialoglycoprotein termed latrophilin.[44]

We observed that anti-GQ1b antibodies induced muscle fiber twitching. This is, however, not a clinical feature of MFS. It might be due to specific properties of NMJs in mouse diaphragm that are not shared by human craniobulbar muscles. The twitching is caused by superimposed MEPPs, just exceeding the firing threshold. As MEPP amplitude is known to vary among different muscles and species, it is likely that anti-GQ1b antibody–induced high-frequency MEPPs will not cause twitching in every type of muscle. In support of this notion we observed that in rat soleus muscle, at which MEPPs are smaller than in mouse diaphragm, MFS1 serum induced high-frequency MEPPs but no twitches (Plomp JJ, unpublished observation). It has been suggested that the vulnerability of craniobulbar nerves in MFS is due to a relatively high concentration of GQ1b, demonstrated in these nerves.[3,45] It is of interest that botulinum toxins, which use gangliosides at NMJs as ectoacceptors, also preferentially affect craniobulbar muscles. Indeed, botulism is one of the major differential diagnoses for MFS and respiratory paralysis is a clinical feature of botulism induced in mice.[15] MFS1 serum had no effects on the electrophysiology of NMJs in intercostal muscle of a nonneurological diseased human subject and also not in mouse flexor digitorum brevis muscles (Plomp JJ, unpublished observations), again suggesting differential sensitivity for anti-GQ1b antibodies among human and murine muscles from different body regions. Furthermore, differential sensitivity for anti-GQ1b antibodies among neurotransmitter release mechanisms at different neuronal cells is suggested from recent studies demonstrating a lack of effect of anti-GQ1b–positive MFS sera on neurotransmitter release from nerve growth factor–differentiated PC12 cells.[46] The reason might be that GQ1b in PC12 cells is not coupled to crucial components of the neurotransmitter release mechanism in the same way as it is at the NMJ.

In view of the lack of effect of MFS1 serum at 32°C on mouse diaphragm, the question arises as to whether NMJs in MFS patients at body temperature are affected at all. However, NMJs in MFS patients are exposed to anti-GQ1b antibodies for a longer period at a higher concentration than NMJs in the in vitro experiments. Furthermore, the temperature dependency of anti-GQ1b antibody–antigen binding may vary among human muscles in such a way that binding to NMJs other than in craniobulbar muscles is prevented at body temperature. Previous studies[17] showed small amplitudes of EPPs and a *reduction* of MEPP frequency in hemidiaphragms after 10 days of passive transfer of Ha rbcM to mice, which differs from the presently found dramatic increase in MEPP frequency after acute incu-

bation with Ha mAb/rbcM. Small EPPs and low MEPP frequency are the electrophysiological features of regenerating NMJs,[23] which possibly were present after 10 days if NMJs were to be damaged in the first few days via an effect, as observed here with in vitro application of Ha mAb/rbcM. On the other hand, the differential effect might be related to suboptimal binding of Ha rbcM to nerve terminals due to a decreased binding capacity of anti-GQ1b antibodies at body temperature.

In conclusion, we have shown that MFS sera induce paralysis in mouse muscle via complement-mediated αLTx-like effects on transmitter release at the NMJ, and our data strongly indicate that anti-GQ1b antibodies are responsible. Furthermore, we suggest that it is likely that anti-ganglioside autoantibodies found in GBS and directed to gangliosides at NMJs other than GQ1b (eg, GM1) might be involved in producing nerve terminal injury through similar mechanisms. Indeed, earlier work has shown that human anti-GM1 antibodies and the GM1 ligand cholera toxin are capable of binding to motor nerve terminals.[26] However, it should be emphasized that none of our data preclude concurrent immunopathological injury to other critical sites in the peripheral nervous system, such as nodes of Ranvier, in either GBS or MFS.

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References

1. Fisher M. An unusual variant of acute idiopathic polyneuritis (syndrome of ophthalmoplegia, ataxia and areflexia). N Engl J Med 1956;225:57–65
2. O'Leary CP, Veitch J, Durward WF, et al. Acute oropharyngeal palsy is associated with antibodies to GQ1b and GT1a gangliosides. J Neurol Neurosurg Psychiatry 1996;61:649–652
3. Chiba A, Kusunoki S, Obata H, et al. Serum anti-GQ1b IgG is associated with ophthalmoplegia in Miller Fisher syndrome and Guillain-Barré syndrome: clinical and immunohistochemical studies. Neurology 1993;43:1911–1917
4. Chiba A, Kusunoki S, Shimuzu T, Kanazawa I. Serum IgG antibody to ganglioside GQ1b is a possible marker of Miller-Fisher syndrome. Ann Neurol 1992;31:677–679
5. Willison HJ, Veitch J, Paterson, Kennedy PGE. Miller-Fisher syndrome is associated with serum antibodies to GQ1b ganglioside. J Neurol Neurosurg Psychiatry 1993;56:204–206
6. Yuki N, Sato S, Tsuiji S, et al. Frequent presence of anti-GQ1b antibody in Fisher's syndrome. Neurology 1993;43:414–417
7. Ledeen RW, Wu G. Ganglioside function in the neuron. Trends Glycosci Glycotech 1992;4:174–187
8. Jacobs BC, Endtz H, Van der Meche FG, et al. Serum anti-GQ1b antibodies recognize surface epitopes on *Campylobacter jejuni* from patients with Miller Fisher syndrome. Ann Neurol 1995;37:260–264
9. Salloway S, Mermel LA, Seamans M, et al. Miller Fisher syndrome associated with *Campylobacter jejuni* bearing lipopolysaccharide molecules that mimic human ganglioside GD3. Infect Immun 1996;64:2945–2949
10. Yuki N, Taki T, Takahashi M, et al. Molecular mimicry between GQ1b ganglioside and liposaccharides of *Campylobacter jejuni* isolated from patients with Fisher's syndrome. Ann Neurol 1994;36:791–793
11. Roberts M, Willison HJ, Vincent A, Newsom-Davis J. Serum factor in Miller-Fisher variant of Guillain-Barre syndrome and neurotransmitter release. Lancet 1994;343:454–455
12. Buchwald B, Weishaupt A, Toyka K, Dudel J. Immunoglobulin G from a patient with Miller-Fisher syndrome rapidly and reversibly depresses evoked quantal release at the neuromuscular junction of mice. Neurosci Lett 1995;201:163–166
13. Buchwald B, Weishaupt A, Toyka K, Dudel J. Pre- and postsynaptic blockade of neuromuscular transmission by Miller-Fisher syndrome IgG at mouse motor nerve terminals. Eur J Neurosci 1998;10:281–290
14. Schwarz A, Futerman AH. The localization of gangliosides in neurons of the central nervous system: the use of anti-ganglioside antibodies. Biochim Biophys Acta 1996;1286:247–267
15. Notermans SHW, Wokke JHJ, Van den Berg LH. Botulism and Guillain-Barre syndrome. Lancet 1992;340:303 (Letter)
16. Rosenthal L, Meldolesi J. Alpha-latrotoxin and related toxins. Pharmacol Ther 1989;42:115–134
17. Willison HJ, O'Hanlon GM, Paterson G, et al. A somatically mutated human antiganglioside IgM antibody that induces experimental neuropathy in mice is encoded by the variable region heavy chain gene, V1–18. J Clin Invest 1996;97:1155–1164
18. Willison HJ, Paterson G, Veitch J, et al. Peripheral neuropathy associated with monoclonal IgM anti-Pr2 cold agglutinins. J Neurol Neurosurg Psychiatry 1993;56:1178–1184
19. Van den Berg RH, Faber-Krol M, Van Es LA, Daha MR. Regulation of the function of the first component of complement by human C1q receptor. Eur J Immunol 1995;25:2206–2210
20. Abraha A, Morgan BP, Luzio JP. The preparation and characterization of monoclonal antibodies to human complement component C8 and their use in purification of C8 and C8 subunits. Biochem J 1988;251:285–292
21. Boelaert J, Joos R, Criel A, et al. C5 deficiency in a white family. Arch Intern Med 1985;145:1333 (Letter)
22. Cruz LJ, Gray WR, Olivera BM, et al. *Conus geographus* toxins that discriminate between neuronal and muscle sodium channels. J Biol Chem 1985;260:9280–9288
23. Di Gregorio F, Fesce R, Cereser S, et al. Spontaneous and nerve-evoked quantal transmission in regenerated motor terminals. Cell Biol Int Rep 1989;13:1119–1126
24. Hong SJ, Chang CC. Use of geographutoxin II (μ-conotoxin) for the study of neuromuscular transmission in the mouse. Br J Pharmacol 1989;97:934–940
25. Plomp JJ, Van Kempen GThH, Molenaar PC. Adaptation of quantal content to decreased postsynaptic sensitivity at single endplates in α-bungarotoxin-treated rats. J Physiol (Lond) 1992;458:487–499
26. O'Hanlon GM, Paterson GJ, Wilson G, et al. Anti-GM1 ganglioside antibodies cloned from autoimmune neuropathy patients show diverse binding patterns in the rodent nervous system. J Neuropathol Exp Neurol 1996;55:184–195
27. Angaut-Petit D, Juzans P, Molgó J, et al. Mouse motor nerve terminal immunoreactivity to synaptotagmin II during sustained quantal transmitter release. Brain Res 1995;681:213–217
28. Hong SJ, Chang CC. Inhibition of acetylcholine release from mouse motor nerve by a P-type calcium channel blocker, ω-agatoxin IVA. J Physiol (Lond) 1995;482:283–290

29. Willison HJ, Veitch J. Immunoglobulin subclass distribution and binding characteristics of anti-GQ1b antibodies in Miller Fisher syndrome. J Neuroimmunol 1994;50:159–165
30. Whaley K. An introduction to the complement system. In: Whaley K, ed. Methods in complement for clinical immunologists. New York: Churchill Livingstone, 1985:1–21
31. Jacobs BC, Endtz HP, Van der Meché FGA, et al. Humoral immune response against *Campylobacter jejuni* lipopolysaccharides in Guillain-Barré and Miller Fisher syndrome. J Neuroimmunol 1997;79:62–68
32. Hurlbut WP, Iezzi N, Fesce R, Ceccarelli B. Correlation between quantal secretion and vesicle loss at the frog neuromuscular junction. J Physiol (Lond) 1990;425:501–526
33. Morgan BP. Physiology and pathophysiology of complement: progress and trends. Crit Rev Clin Lab Sci 1995;32:265–298
34. Turner MW. The lectin pathway of complement activation. Res Immunol 1996;147:110–115
35. Nicholson-Weller A, Halperin JA. Membrane signalling by complement C5b-9, the membrane attack complex. Immunol Res 1993;12:244–257
36. Van der Kloot W, Molgó J. Quantal acetylcholine release at the vertebrate neuromuscular junction. Physiol Rev 1994;74:899–991
37. Koski CL, Sanders ME, Swoveland PT, et al. Activation of terminal components of complement in patients with Guillain Barré syndrome and other demyelinating neuropathies. J Clin Invest 1987;80:1492–1497
38. Hartung HP, Schwenke C, Bitter-Suermann D, Toyka KV. Guillain-Barré syndrome: activated complement components C3a and C5a in CFS. Neurology 1987;37:1006–1009
39. Hafer-Macko C, Hsieh ST, Li CY, et al. Acute motor axonal neuropathy: an antibody-mediated attack on axolemma. Ann Neurol 1996;40:635–644
40. Hafer-Macko C, Sheikh KA, Li CY, et al. Immune attack on the Schwann cell surface in acute inflammatory demyelinating polyneuropathy. Ann Neurol 1996;39:625–635
41. Valtorta F, Maddedu L, Meldolesi J, Ceccarelli B. Specific localization of the α-latrotoxin receptor in the nerve terminal plasma membrane. J Cell Biol 1984;99:124–132
42. Petrenko AG, Perin MS, Davletov BA, et al. Binding of synaptotagmin to the α-latrotoxin receptor implicates both in synaptic vesicle exocytosis. Nature 1991;353:65–68
43. Krasnoperov VG, Bittner MA, Beavis R, et al. α-Latrotoxin stimulates exocytosis by the interaction with a neuronal G-protein-coupled receptor. Neuron 1997;18:925–937
44. Davletov BA, Shamotienko OG, Lelianova VG, et al. Isolation and biochemical characterization of a Ca^{2+}-independent α-latrotoxin-binding protein. J Biol Chem 1996;271:23239–23245
45. Chiba A, Kusunoki S, Obata H, et al. Ganglioside composition of the human cranial nerves, with special reference to pathophysiology of Miller Fisher syndrome. Brain Res 1997;745:32–36
46. Benetar MG, Willison HJ, Vincent A. Lack of effect of Miller Fisher sera/plasmas on transmitter release from PC12 cells. J Neuroimmunol 1997;80:1–5