Gangliosides and anti-ganglioside antibodies in neuromuscular synaptic function

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CHAPTER 3.4

Neuropathophysiological potential of anti-ganglioside complex sera at mouse neuromuscular junctions

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Abstract

Some Guillain-Barré syndrome patients have serum antibodies against antigenic epitopes formed by a complex of two different gangliosides but direct evidence for pathogenicity is lacking. Here we show that anti-GM1/GD1 and anti-GM1/GQ1b complex-positive sera can induce functional damage at motor nerve terminals of mouse neuromuscular junctions *ex vivo*, similar to effects shown by us earlier of antibodies against single gangliosides. However, only about half of the 27 investigated anti-ganglioside complex sera induced neuropathogenic effects, with high variability in potency, and requiring high antigenic density. These results suggest antigenic presence of ganglioside complexes in living neuronal membranes and show pathogenic roles for some anti-ganglioside complex antibodies.

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Introduction

With the current methods, anti-ganglioside antibodies are detected in 50-60% of patients with Guillain-Barré syndrome (GBS) (Kusunoki et al., 2008; Notturno et al., 2009; Van Doorn et al., 2008). Gangliosides are a family of sialic acid-containing amphiphilic sphingolipids that are enriched in neuronal membranes. Anti-ganglioside antibodies are thought to be pathogenic, either by disturbing neuronal functions of gangliosides or by mediating complement activation, thereby damaging peripheral nerve axons and possibly also motor nerve terminals at the neuromuscular junction (NMJ) in some cases (Van Doorn et al., 2008; Plomp and Willison, 2009). Recently, it has been suggested that combinations of two different gangliosides can form a novel antigenic epitope and that some GBS patients have antibodies against such a complex (Kusunoki et al., 2008; Kaida et al., 2004a). Clinical correlation and fine-specificity studies estimate that 10-20% of GBS patients has anti-ganglioside complex antibodies (Notturno et al., 2009; Kaida et al., 2007; Kuijf et al., 2007). Interestingly, antibodies against GD1a/GD1b and GD1b/GT1b complexes seem associated with disease severity (kaida et al., 2007), although this not confirmed by others (Notturno et al., 2009). In the GBS variant Miller Fisher syndrome (MFS), associated with anti-GQ1b ganglioside antibodies, the incidence of anti-complex antibodies seems higher: a study by Kaida and colleagues showed that 7 of 12 MFS sera contained antibodies against a complex of at least GQ1b or GT1a and another ganglioside (kaida et al., 2006).
The observations that anti-ganglioside complex antibodies disappear upon clinical recovery (Kuijf et al., 2007) and their association with disease severity (Kaida et al., 2007) support a neuropathophysiological role. However, this has not yet been directly shown. We here performed a first investigation of 17 GBS sera with anti-GM1/GD1a complex activity and 10 GBS variant sera with anti-GM1/GQ1b complex activity for their ability to induce complement-dependent deleterious effects at mouse diaphragm motor axon terminals. Earlier, we showed that antibodies against the single ganglioside species bind to and induce structural and functional lesions at this site, electrophysiologically hallmarked by a high frequency of miniature endplate potentials (MEPPs, the postsynaptic responses to uniquantal acetylcholine release), causing asynchronous muscle fibre twitches and, eventually, block of neurotransmission (Plomp and Willison, 2009).

Material and methods

Patient sera and mouse monoclonal antibodies
Acute-phase sera were obtained from 27 GBS and GBS-variant patients from Japan (# 5-15 and 18-27), The Netherlands (# 1-4) and Bangladesh (# 16-17) and stored at -80 °C. Normal human serum (NHS) from a healthy donor was stored aliquoted at -80 °C. Mouse monoclonal antibodies (mAbs) against GQ1b (CGM3; 50 μg/ml), GD1a (MOG35; 100 μg/ml) and GM1 (DG2; 100 μg/ml) were used as positive controls (Goodfellow et al., 2005; Goodyear et al., 1999). Prior to experimental use, all sera and mAbs were dialyzed overnight at 4 °C against Ringer's solution (116 mM NaCl, 4.5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM NaH_2PO_4, 23 mM $NaHCO_3$, 11 mM glucose, pH 7.4), pre-gassed with 95% O_2 /5% CO_2.

Enzyme-linked immunosorbent assay
Sera (1:100) were tested, as described (Kuijf et al., 2007), for IgM and IgG antibody activity against individual gangliosides and complexes, as indicated in the Results. Serum scoring an optical density (OD) of >0.2 was considered positive. For anti-ganglioside-complexes, positivity was defined as having an OD of >0.2 higher than the highest OD for an individual ganglioside (Kaida et al., 2004; Kuijf et al., 2007). All samples were tested in duplicate.

Bioassays at mouse hemi-diaphragm-phrenic nerve preparations
Male and female wildtype and GD3synthase knockout (GD3s-KO) mice 11 were used at 1-4.5 months of age. Hemi-diaphragms with phrenic nerves were dissected and kept in Ringer's medium at room temperature (20-22 °C). Muscles were incubated with 33% GBS heat-inactivated (30 min at 56 °C to destroy complement) serum in Ringer's medium or mouse mAbs for 3 h at 32 ºC, rinsed in Ringer's medium for 10 min and exposed to 33% NHS in Ringer's medium for 1 h at room temperature. Micro-electrode recording of MEPPs (10-30 NMJs per session) and visual scoring of spontaneous asynchronous fibre twitching (0 for no twitching across the hemi-diaphragm, 1 for twitching of <10 fibres, 2 for a small amount, 3 for a moderate amount and 4 for an extensive amount) were done as described (Zitman et al., 2008; O'Hanlon et al., 2001). Depending on the available volumes, sera were tested 1 to 4 times and the mean values of the parameters were calculated. Animal experiments were carried out according to Dutch law and Leiden University guidelines.

Complement immunohistochemistry
C3 deposition at NMJs was quantified in a selection of the electrophysiologically tested samples, as described (Greenshields et al., 2009).

Results

Testing of sera for anti-ganglioside-complex specificity
Sera were selected on the basis of their positivity for anti-ganglioside complexes (GM1/GQ1, GM1/GD1a or GD1a/GD1b), as had been determined at their original research centre. In addition, the Japanese sera had also been tested in Japan for activity against GT1a, GD3, GalNAc-GD1a, and GA1 and ganglioside complexes GM1/GalNAc-GD1a and GD1b/GT1b. In view of possible inter-laboratory variations (Willison et al., 1999), the complete series was re-tested in Rotterdam (Erasmus MC) in a single experimental ELISA run for IgG and IgM antibodies against gangliosides GM1, GQ1b, GD1a and GD1b and ganglioside complexes GM1/GQ1b, GM1/GD1a, GM1/GD1b, GD1a/GD1b. On the basis of these ELISA studies, we classified sera into two categories (Table 3.1): 1) sera that tested positive for GM1/GD1a ganglioside complex (17 sera: # 1-17) and 2) sera that tested positive for GM1/GQ1b ganglioside complex (10 sera: # 18-27). One serum (# 26) was found negative for anti-GM1/GQ1b complex when re-tested but was still included in the second group because it had been found positive in Japan. Most sera of both categories showed additional activities against either the individual gangliosides of the complex for which they were positive, or against other individual gangliosides or other complexes (Table 3.1).

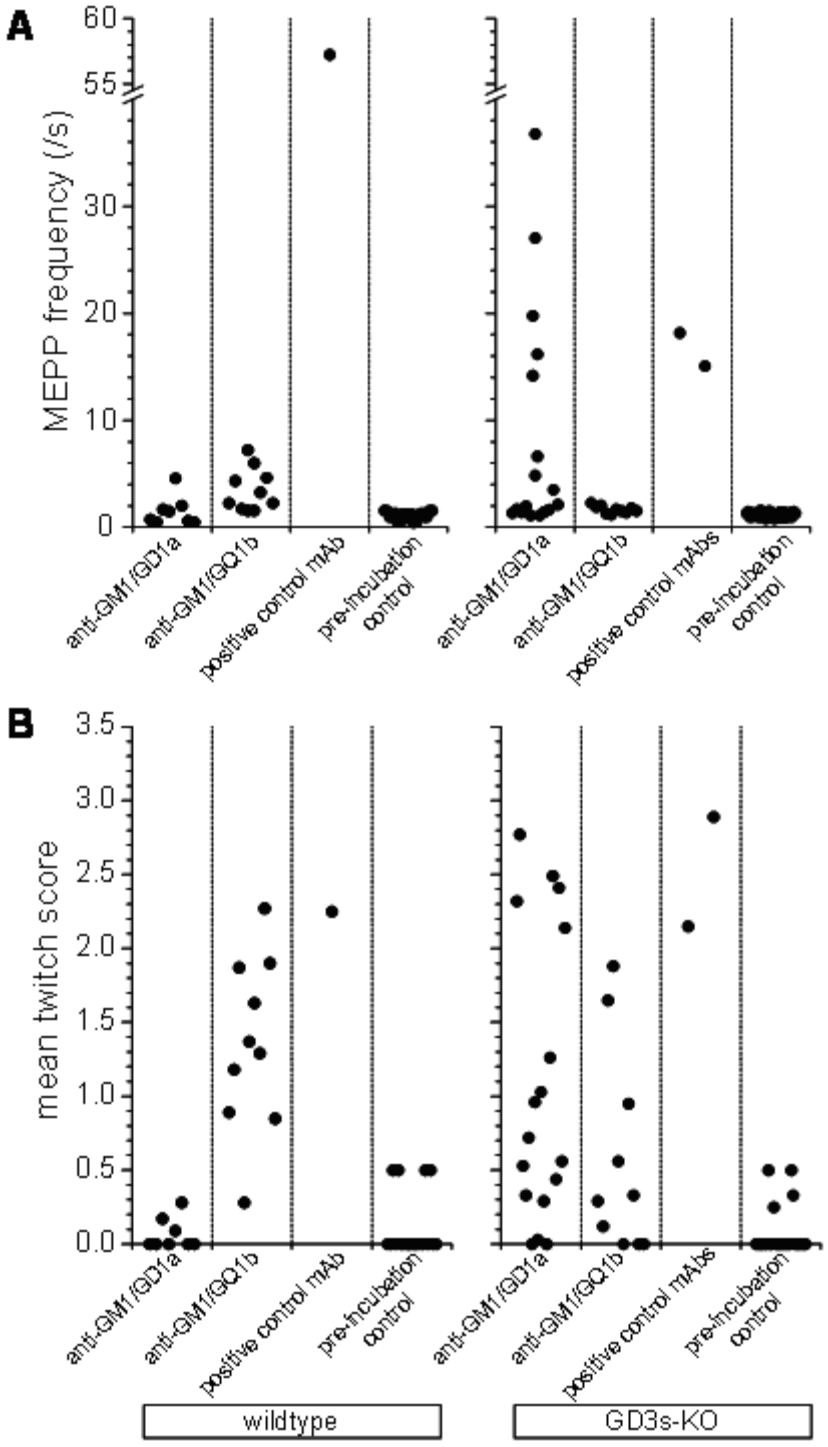

Figure 3.15.
A. Effect of anti-ganglioside complex sera on MEPP frequency at wildtype (left panel) and GD3s-KO (right panel) mouse diaphragms NMJs. Eight of the 17 tested anti-GM1/GD1a-complex sera induced MEPP frequency increases at GD3s-KO to a level of more than twice the pre-incubation control value. Only one serum (modestly) increased MEPP frequency at wildtype NMJs. Of the 10 investigated anti-GM1/GQ1b complex sera, 5 induced (modest) MEPP frequency rise to levels of more than twice the control value, exclusively at wildtype NMJs. **B.** Effect of anti-ganglioside complex sera on muscle fibre twitches at wildtype (left panel) and GD3s-KO (right panel) mouse NMJs. Most anti-ganglioside-complex sera that induced MEPP frequency elevation scored higher than 1 for muscle fibre twitching upon visual inspection.

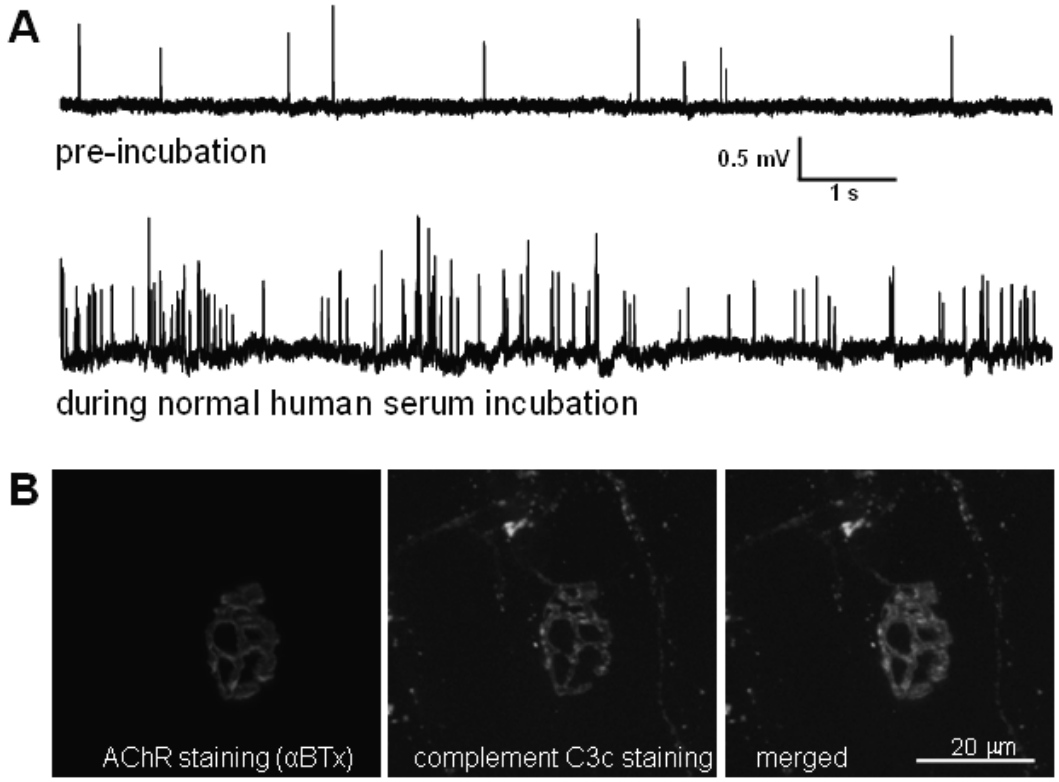

Figure 3.16.
Examples of MEPPs recorded before incubation with heated anti-GM1/GD1a-complex serum # 7 (upper panel in **A**) and during subsequent incubation with normal human serum as complement source (lower panel in **A**). Sweep length is 10 s. **B.** Example of C3c immunostaining (middle panel) at an NMJ (defined by fluorescently labelled α-bungarotoxin (αBTx) binding to acetylcholine receptors [AChR], left panel) from a muscle preparation that had been exposed to anti-GM1/GD1a complex serum # 14 and normal human serum and had shown elevated MEPP frequency and clear muscle fibre twitches.

Pathophysiological effects at mouse neuromuscular synapses

At NMJs of GD3s-KO mice (used because their membranes express upregulated density of GM1 and GD1a 15) 8 of the 17 anti-GM1/GD1a complex sera induced variable elevations of mean MEPP frequency (range 3.5-36.8 /s, the control pooled mean value before incubations was 1.2 /s; Figure 3.15 and Table 3.1) during the period of NHS incubation. From 8 of the 17 sera, sufficient material was present to also study their effects at wildtype NMJs: only one serum (# 1) showed moderate elevation of MEPP frequency (to 4.6 /s). In the anti-GM1/GQ1b series, elevated MEPPs were found for 5 of the 10 tested sera (range 3.3-7.2 /s) at wildtype NMJs. No effect was found at GD3s-KO NMJs (Figure 3.15).

Elevated MEPP frequency generally was accompanied by muscle fibre twitching, as visually scored (range 0-4) every 5 min during the 1 h NHS incubation period (Figure 3.16 and Table 3.1). Average twitching score was <0.5 in the control (pre-incubation) session. Similar scores were obtained with the GM1/GD1a series sera when tested in wildtype (range 0.0-0.3). In GD3s-KO muscles 7 of these sera scored >1.0 (Figure 3.16) with highly variable scores (range 1.0-2.8). Seven of the 10 GM1/GQ1b series

sera scored >1.0 in wildtype muscles (range 1.2-2.3). At GD3s-KO muscles, 2 of the 10 GM1/GQ1b sera scored >1.0 (1.7 and 1.9). The mean score of positive control mAbs was 2.2 or higher (Figure 3.16).

Complement deposition at the neuromuscular synapse
For the group of anti-GM1/GD1a sera, deposited amounts of complement component C3c at NMJs were found to be associated with levels of elevated MEPP frequency ($p<0.01$, Spearman's rank correlation). In the anti-GM1/GQ1b series, NMJ C3c deposition was generally low and not consistently associated with MEPP frequency level (Table 3.1).

Discussion

The special category of anti-ganglioside complex antibodies in GBS sera was discovered by Kaida and colleagues in ELISA studies (Kaida et al., 2004a). However, no direct evidence of a pathogenic action at living neuronal membranes has been provided yet. We here report the potency of sera containing either anti-GM1/GD1a or anti-GM1/GQ1b antibodies to produce pathophysiological effects at mouse NMJs. Our earlier studies at (*ex vivo* and *in vivo*) mouse NMJs demonstrated the damaging effects of patient sera, purified antibody or mouse mAbs with (ELISA-defined) activity against either single gangliosides GQ1b, GD1a, GM1 and GD1b (for review see Plomp an Willison, 2009). These presynaptic effects were complement-dependent and electrophysiologically hallmarked by a high frequency of MEPPs, sometimes triggering a muscle fibre contraction. Roughly half of the anti-ganglioside-complex sera tested in the current *ex vivo* experiments induced such effects, albeit at rather moderate level. This indicates that anti-ganglioside complex antibodies are in principal capable of binding to live neuronal membranes and, by activating complement, can induce pathophysiological effects. Thus, these antibodies are likely of pathogenic relevance, as already suggested from the clinical association with specific patterns of paralysis (in particular cranial nerve involvement) and, in some patient groups, the requirement of mechanical ventilation (Van Doorn et al., 2008; Kaida et al., 2007). Our findings at mouse NMJs suggest that paralysis in anti-ganglioside complex antibody-positive patients may, besides axonal dysfunction, also involve some degree of NMJ synaptopathy.
However, our results are not unambiguous. First, not all anti-ganglioside complex-positive sera induced the deleterious effects at mouse NMJs. Second, the level of the effect caused by the active sera was only moderate as compared with that of the positive control mouse mAbs as well as with earlier tested patient sera with activity against single ganglioside species (Goodfellow et al., 2005; Greenshields et al., 2009; Plomp et al., 1999). These two inconsistencies may relate to variability in titre and affinity of the pathogenic antibodies amongst sera in combination with the likely existence of an antibody binding threshold for the induction of pathophysiological effects at NMJs. Third, the effects of anti-GM1/GD1a sera were only found at NMJs of GD3s-KO mice and not at those of wildtypes, indicating the requirement of high density of the targeted ganglioside complexes, possibly indicating that human patients have a special predisposing ganglioside configurations in their neuronal membranes. Fourth, many sera, especially in the GM1/GD1a series, had various additional

activities against single types of gangliosides and some sera even had additional activity against different complexes (Table 3.1). This complicates the interpretation of the result, in particular because the two monospecific anti-GM1/GD1a sera (# 16 and 17) lacked pathophysiological effects. This might suggest that besides anti-GM1/GD1a complex activity, another activity must be present in order to have enough antibody binding to exert effects. It is even not completely excluded that either antibodies against GM1a or GD1a were solely responsible for inducing effects, because all MEPP frequency-elevating anti-GM1/GD1a-positive sera were also positive for antibodies against at least one of these two individual gangliosides. On the other hand, there were also non-MEPP frequency elevating sera that contained activity against either GM1, GD1a, or both. Activity against single gangliosides was less of a problem in the GM1/GQ1b group where 2 of the 6 monospecific anti-ganglioside complex sera induced effects at wildtype NMJs and not at GD3s-KO NMJs (which contain no GQ1b; Figure 3.15), making it highly likely that the anti-ganglioside complex antibodies were responsible. However, in this series, the effects were rather modest in magnitude and complement deposition did not very well correlate with elevation of MEPP frequency, further complicating matters.
In conclusion, it is clear that a proportion of the anti-ganglioside complex sera has neuropathophysiological effects at mouse NMJ, suggesting a pathogenic role in GBS patients, but there are also some difficulties in the interpretation of these results at this stage. Development of high-affinity mouse mAbs with monospecific activity against defined ganglioside complexes is needed to allow a more detailed and extensive study of the deleterious effects of anti-ganglioside complex antibodies.

Table 3.1. (next page)
Anti-ganglioside (IgG or IgM) activity, effects on MEPP frequency (fMEPP), muscle fibre twitching and C3c deposition at neuromuscular junctions of sera with positivity for either anti-GM1/GD1a antibodies (sera # 1-17) or anti-GM1/GQ1b antibodies (sera # 18-27).
Columns anti-ganglioside activity: - : negative.
Columns fMEPP: - : < twice the control mean; + : > twice the control mean and <10 /s; ++ : between 10 and 20 /s; +++ : >20 /s; empty: not done. Elevated MEPP frequencies are also highlighted.
Columns twitching: - : <1.0; + : between 1.0 and 2.0; ++ : more than 2.0; empty: not done.
C3c staining was done in one experimental run on 17 tissue samples. Indicated is the relative intensity within this series: + : low; ++: moderate; +++ : high; empty: not done.

positive for ganglioside complex		activity against other single gangliosides or complexes				GD3s-KO mouse tissue			wildtype mouse tissue		
						pathophysiology		C3c	pathophysiology		C3c
	serum#	GM1	GD1a	GQ1b	other	fMEPP	twitching	staining	fMEPP	twitching	staining
GM1/GD1a	1	-	yes	-	-	+	+		+	-	++
	2	yes	yes	-	-	-	-		-	-	+++
	3	yes	yes	-	-	-	-		-	-	
	4	yes	-	-	-	+	+		-	-	+
	5	-	-	yes	GD1b, GM1/GD1b	-	+				
	6	-	-	-	GalNAc-GD1a, GM1/GalNAc-GD1a	-	+				
	7	yes	yes	-	GD1b	++	+++	++			
	8	-	yes	yes	GD1b, GT1a	-	-	+			
	9	yes	-	-	GD1b, GA1	+	-				
	10	yes	-	-	GD1b, GM1/GD1b	-	+	+			
	11	yes	yes	-	GD1b , GalNAc-GD1a	++	+++	++			
	12	-	yes	-	GD1a/GD1b, GD1b/GT1b	+++	+++	+++	-	-	
	13	yes	-	-	GD1b, GD1a/GD1b, GD1b/GT1b	-	+		-	-	
	14	yes	yes	-	GD1b, GD1a/GD1b, GD1b/GT1b	+++	+++	+++	-	-	
	15	yes	yes	-	GD1b, GD1a/GD1b, GD1b/GT1b	++	+++	+++	-	-	
	16	-	-	-	-	-	-	+			
	17	-	-	-	-	-	-				
GM1/GQ1b	18	-	-	-	-	-	-		-	++	
	19	-	-	-	-	-	+		-	++	
	20	-	-	yes	-	-	+		+	++	
	21	yes	-	-	-	-	++		-	-	
	22	-	-	-	-	-	-	+	+	+++	++
	23	-	-	-	-	-	++		+	++	
	24	-	-	yes	GT1a	-	-	+	+	++	++
	25	-	-	-	-	-	-		-	++	
	26	-	yes	-	-	-	-	+	+	+	+
	27	-	-	-	-	-	-		-	+	