Neurochemical Research, Vol. 27, Nos. 7/8, August 2002 (© 2002), pp. 697–709

Complex Gangliosides as Autoantibody Targets at the Neuromuscular Junction in Miller Fisher Syndrome: A Current Perspective*

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(Accepted March 21, 2002)

Glycosphingolipid biology has increasingly interfaced with the field of human autoimmune neuropathy over the last two decades. There are currently over 20 distinct glycolipids that have been identified as autoantibody targets in a wide range of clinical neuropathy syndromes. This review sets out the clinical and experimental background to one interesting example of anti-glycolipid antibody-associated neuropathy termed Miller Fisher syndrome. This syndrome, comprising the triad of ataxia, areflexia, and ophthalmoplegia, correlates highly with the presence of serum anti-GQ1b antibodies, arising through molecular mimicry with microbial oligosaccharides. Anti-GQ1b antibodies mediate neural injury through binding to GQ1b-enriched sites in the peripheral nervous system, including extraocular nerves. Animal experimental evidence, along with a hypothetical background, indicates the motor nerve terminal may be a key site for anti-GQ1b antibody binding with consequent defects in synaptic transmission, as occurs in botulism and other toxinopathies. Our work in recent years on this hypothesis is summarized.

KEY WORDS: Gangliosides; GQ1b; autoantibodies; Miller Fisher syndrome; Guillain Barré syndrome; peripheral neuropathy; motor nerve terminal; neuromuscular junction; synaptic transmission.

INTRODUCTION

Over the last two decades, ganglioside and glycolipid biochemistry has increasingly overlapped with the field of human autoimmune neuropathy. It is now evident that over 20 different glycolipids act as autoantibody targets in a highly diverse group of neuropathies with distinct clinical phenotypes (1–3). In this review we will specifically focus on one highly interesting area we have extensively researched in recent years, namely the emerging relationship between motor nerve terminal injury, complex gangliosides, anti-GQ1b antibodies, and the Miller Fisher syndrome (MFS) (4).

Much progress has been made over the last decade in our understanding of the pathogenesis of MFS, a syndrome that comprises the clinical triad of ataxia, areflexia and ophthalmoplegia (5). Although MFS has long attracted attention as an infrequent clinical curiosity, the renaissance of interest in MFS began in 1992 when Chiba and colleagues (6) reported the presence of anti-GQ1b antibodies in the acute phase serum of MFS cases, a finding that has been substantiated and extended in many subsequent studies (see below).

* Special issue dedicated to Dr. Guido Tettamanti.

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0364-3190/02/0800–0697/0

The strength of this clinical-serological association is such that the measurement of anti-GQ1b antibodies in suspected cases of MFS is of diagnostic use to clinicians. Furthermore, extensive efforts have been made to understand the pathophysiological basis of the paralysis arising in this syndrome of which the motor manifestations are very specifically localised to the extraocular and lower cranial musculature. In this respect, it shows a remarkable similarity to the sites clinically affected at the onset of human botulism, a known disorder of presynaptic transmitter release at the neuromuscular junction (NMJ).

The similarity with botulism led us to focus on the presynaptic nerve terminal as a target site for anti-GQ1b antibody mediated injury. Many bacterial toxins, including botulinum, tetanus and cholera toxins bind to the ganglioside-enriched nerve terminal and are subsequently taken up into the NMJ (7–9). The NMJ may be particularly vulnerable to antibody-mediated attack in MFS as it lies outside the blood-nerve barrier. In support of this, the NMJ is also the pathogenic site for other well-recognized antibody-mediated autoimmune diseases, including myasthenia gravis and myasthenic syndromes. There is also some clinical electromyographic evidence to suggest that the nerve terminal may be injured in some cases of MFS (10,11).

The Clinical and Serological Features of Miller Fisher Syndrome

MFS is a clinical variant of Guillain-Barré syndrome (GBS), the acute postinfectious paralytic illness caused by inflammatory disruption of peripheral nerve integrity and function (12,13). In contrast to the generalised and often severe limb, respiratory and axial weakness and sensory loss that occurs in GBS, the manifestations of MFS are curiously restricted to limb ataxia, tendon reflex loss and extraocular muscle paralysis. Many cases also show paralysis of lower cranial muscles subserving facial movement, speech and bulbar function. MFS has this feature in common with botulism, with which it may be clinically confused. Affected cases usually make a good clinical recovery and MFS is relatively rare, accounting for 5–10% of GBS cases, the incidence of the latter syndrome being 1–2 per 100,000 (14). MFS has emerged as the archetypal anti-ganglioside antibody-mediated human neuropathy and is providing valuable insights into the pathogenesis of its more serious counterpart, GBS (4).

The presence of anti-GQ1b ganglioside antibodies in sera of patients with MFS and a cluster of closely related syndromes which have in common the presence of ophthalmoplegia is well documented (6,13,15–18). In MFS, serum anti-ganglioside antibody titres are at their peak at clinical presentation and decay rapidly in most cases concomitant with clinical recovery, being undetectable as early as 1 month after onset (19,20). Equally significant is the complete absence of anti-GQ1b antibodies from normal and disease control groups, indicating a high level of specificity for MFS and related diseases.

Anti-GQ1b antibodies almost invariably cross-react with the structurally similar ganglioside, GT1a, although occasional exceptions exist (21,22). Up to 50% of MFS sera also demonstrate reactivity with other gangliosides containing a disialosyl epitope, such as GD3, GD1b, and, occasionally, GT1b (23). Relevant ganglioside structures are shown in Figure 1. A number of recent reports suggest that GQ1b monospecific antibodies are more strongly associated with opthalmoplegia, whereas GT1a monospecific antibodies are associated with oropharyngeal palsy, although these are most likely relative rather than absolute findings (24–27).

The anti-GQ1b antibodies that occur in MFS are polyclonal, and of IgM, IgA, and IgG classes, the latter being the most commonly encountered and measured in clinical diagnostic practice (19). Unusual for carbohydrate antigens, the IgG response is typically restricted to the IgG_1 and IgG_3 subclasses suggesting T cell help has been recruited. This is important because human IgG_1 and IgG_3 are usually of high affinity and are potent activators of the complement system.

Of interest is a chronic neuropathy in which serum IgM paraproteins react with NeuAc(α2-8)NeuAc(α2-3) Gal–configured disialosyl epitopes common to many of the MFS-associated gangliosides including GD1b, GD3, GT1a, GT1b, and GQ1b (28). The clinical picture is highly reminiscent of MFS, and comprises a chronic neuropathy with marked sensory ataxia and areflexia,

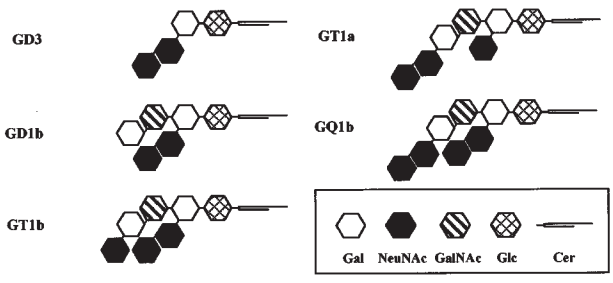

Fig. 1. Structures of gangliosides associated with autoantibodies in Miller Fisher syndrome.

relatively preserved motor function in the limbs and in some cases weakness affecting oculomotor and bulbar muscles as fixed or as relapsing-remitting features. We have used serum and purified monoclonal antibodies from these cases to investigate motor nerve terminal dysfunction, as described below.

The temporal pattern of clinical onset, nadir, and spontaneous recovery that occurs in MFS is highly suggestive of an acute phase primary immune response, peaking 10–14 days after an infectious event, followed by gradual decay of the immune response (29). Indeed, MFS follows a wide variety of infections including *Campylobacter jejuni (C. jejuni)* enteritis and viral and bacterial infections of the upper respiratory tract. It is now evident that molecular mimicry (the sharing of structurally homologous antigenic determinants) between GQ1b/GT1a and *C. jejuni* lipopolysaccharide (LPS) is central to the induction of this response (1,30–34). *C. jejuni* isolates from MFS cases have been studied by spectroscopic analysis and immunodetection methods using human and murine antibodies and found to contain GD3- and GT1a-like oligosaccharides in their LPS core oligosaccharides (35–37). Furthermore, we have shown that immunisation of the mouse with GT1a containing LPS can produce a serum anti-GQ1b antibody response, and it has been possible to derive monoclonal antibodies from these mice that react with GQ1b, GT1a, GD3, and other disialylated gangliosides (38).

Ganglioside Distribution and the Site Specificity of Paralysis in MFS

Restriction of the paralytic effects of MFS to a limited group of muscles may be due to the patterns of ganglioside distribution within cranial and somatic nerves. Chiba and colleagues first noted using immunohistology that the extraocular cranial nerves expressed high levels of GQ1b at nodes of Ranvier (21). Furthermore, they showed biochemically that the nerve trunks supplying the human extraocular muscles have a relatively high content of GQ1b compared with other cranial or spinal nerves (39). However, in the same study, GQ1b was also present in significant amounts at sites unaffected by MFS, and we have shown that MFS-like anti-ganglioside antibodies bind to the nodes of Ranvier in other nerves (28,38). Thus, although of major importance, the absolute tissue distribution (i.e., presence or absence) of particular gangliosides is unlikely to be the sole explanation for regional localisation of the clinical pathology. Another factor may be related to the molecular arrangements and function of gangliosides in neurons. Within phospholipid membranes, gangliosides are enriched in microdomains or "lipid rafts," into which proteins such as growth factor receptors or ion channels are specifically included or excluded (40) and it appears that gangliosides can be intimately involved in the normal functioning of such proteins (41,42). Bivalent IgG and multivalent IgM antibodies may be more readily able to bind with high avidity to clusters of gangliosides in rafts, than to gangliosides evenly distributed throughout membranes.

Immunohistological analyses of the NMJ, including the specialized *en grappe* end-plates of polyinnervated muscle fibres found within the extraocular muscle, have demonstrated the binding of antibodies reactive to polysialylated gangliosides (4,28,38,43). In addition to motor involvement, the vast majority of MFS cases also have sensory ataxia and reflex loss, suggesting that GQ1b or GQ1b-like antigens may be expressed in other neural sites, such as cerebellar neurons, dorsal root ganglia and muscle spindles, as discussed in detail elsewhere (4).

As well as structural and anatomical factors, the accessibility of target gangliosides to circulating antibodies may be important for the induction of paralysis. The NMJ is particularly vulnerable to antibody-mediated attack in MFS and other nerve terminal disorders as it lies outside the blood-nerve barrier. This does not preclude the likelihood that the node of Ranvier on oculomotor nerves is another site of anti-GQ1b antibody mediated injury (21); however, the node of Ranvier is protected by the blood nerve barrier in a way that may limit antibody access. Similarly, an explanation for the rarity of CNS involvement in MFS is the protection from autoimmune attack afforded by the preserved blood-brain barrier, rather than the absence of GQ1b in CNS neural membranes.

Finally, the activity of inflammatory mechanisms that are triggered by antibody binding, such as complement activation and complement regulators needs to be considered. Thus, some neuronal or glial membranes may be more susceptible to low grade complement mediated attack than others, in part dependent upon the distribution of complement regulatory proteins, such as decay accelerating factor and CD59 (44).

Synaptic Transmission at the Neuromuscular Junction

In our studies on the electrophysiological effects of MFS sera and anti-ganglioside mAbs, we have mainly used the mouse phrenic nerve hemidiaphragm

preparation. This muscle is very suitable for in vitro electrophysiological measurements because it is thin and flat and has a well-defined midline region within which the NMJs are localized. Furthermore, it is easy to dissect, with the attached phrenic nerve being of sufficient length to be placed on a bipolar electrode for stimulation.

Prior to describing our findings, we will briefly review normal NMJ physiology and the electrophysiological observations one can make in this model system. Neurotransmission at the mammalian NMJ involves a cascade of events in which presynaptic neuro-exocytotic proteins and many types of pre- and postsynaptic ion channels come into play. The axon motor nerve impulse is conducted passively into the most terminal axonal branches. There, it induces the brief opening of P-type voltage-gated Ca^{2+} channels (45). These Ca^{2+} channels are localized at active zones and presumably form complexes with components of the neuro-exocytotic machinery, such as SNAP-25, syntaxin, and synaptotagmin (46). For a review on components and function of the neuro-exocytotic machinery, see (47). The influx of Ca^{2+} results in a large rise of cytosolic [Ca^{2+}] which stimulates pre-docked synaptic vesicles to fuse with the presynaptic membrane and liberate their content of about 10,000 molecules of acetylcholine (ACh; the "quantum") into the synaptic cleft. Some of the released ACh is hydrolysed by extra-cellular cholinesterase located within the cleft, and the remainder binds to postsynaptic receptors (AChRs). These are heteromultimeric ligand-gated ion channels localized in extremely high density at the tops of the postsynaptic junctional folds on the muscle fibre membrane. AChR opening allows for simultaneous inflow of Na^+ and outflow of K^+ ions. The net inward flow of electrical current results in a local depolarization of the muscle fibre membrane, the so-called endplate potential (EPP), which is 15–40 mV in amplitude, depending on various factors such as muscle type, species and age. The EPP triggers an action potential that spreads out on the muscle fibre membrane and further into the T-tubular system where it initiates a sequence of intracellular events that finally results in fibre contraction.

The NMJ is highly reliable in its task; under normal conditions every motor nerve impulse results in muscle fibre contraction. In order to achieve such robustness, more ACh is released then strictly necessary to trigger a muscle action potential. An excellent review on this "safety factor" phenomenon has recently been published (48).

Besides nerve action potential-evoked ACh release, there is spontaneous release of single ACh quanta from motor nerve terminals, resulting in the generation of a miniature endplate potential (MEPP), a small postsynaptic depolarization of about 0.5–1 mV. The frequency and amplitude of such uniquantal events depend on several factors and vary between species and muscle types, as well as with age. Individual MEPPs are normally too small to trigger a muscle fiber action potential and their possible physiological role is unclear.

With in vitro electrophysiological techniques, ACh release can be measured at NMJs in nerve/muscle preparations (Fig. 2). Upon impalement of a muscle fiber with the ultrafine tip of a glass microelectrode connected to standard electrophysiological equipment, MEPPs and EPPs can be recorded (49). In order to eliminate muscle action potentials, and thus contractions that hamper the microelectrode measurements, the pharmacological tool μ-Conotoxin GIIIB can be used, which specifically blocks muscle fiber Na^+ channels (50). As a result, the EPPs, normally masked by the muscle action potential they trigger, are revealed. From the amplitudes of EPP and MEPP at a NMJ, the quantal content can be calculated, i.e. the number of ACh quanta that was released and caused the EPP.

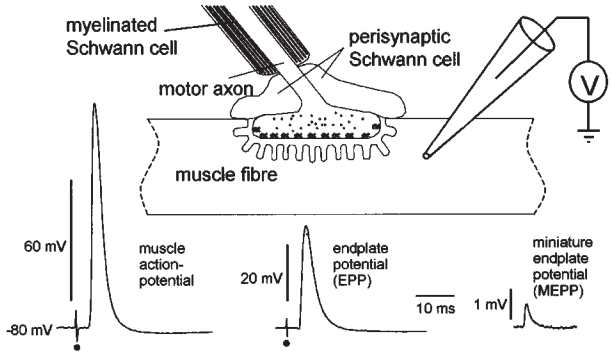

Fig. 2. Electrophysiological analysis of the neuromuscular junction in mouse diaphragm ex vivo. In a muscle fiber that is impaled by a voltage-sensing microelectrode near the endplate, a resting membrane potential of around −80 mV can be measured. Examples of the synaptic signals that can be recorded are shown. Miniature end-plate potentials (MEPPs) are small depolarizations of about 1 mV that occur spontaneously at a rate of about 0.5/s in diaphragm neuromuscular junction of young mice. The MEPP is the result of the presynaptic release of a single ACh quantum, liberated from one synaptic vesicle by exocytosis. Electrical stimulation of the nerve trunk of the nerve-muscle preparation (at time point indicated with black dot and visible as an artefact on the voltage trace) triggers an action potential that invades the nerve terminal and stimulates the simultaneous release of a number of ACh quanta. The postsynaptic effect is a large depolarization (15–40 mV), the endplate potential (EPP), that normally will trigger an action potential in the muscle fiber which subsequently triggers contraction. To be able to record the EPP without being hampered by the action potential it triggers, μConotoxin GIIIB is used. This toxin selectively blocks Na^+ channels of the muscle membrane. From the amplitudes of EPP and MEPP, the quantal content, i.e., the number of ACh quanta that was released upon a nerve impulse, can be calculated.

Electrophysiological Effects of MFS Sera and Anti-GQ1b Monoclonal Antibodies

For the reasons outlined earlier, we decided to study the effects of MFS sera and related monoclonal anti-ganglioside antibodies (mAbs) on NMJ electrophysiology. Whilst acknowledging the detailed work of other groups on this topic (51,52), we will limit this discussion to our own published findings and some recent additional unpublished results. We tested the effects of incubation of mouse hemi-diaphragm preparations with a series of nine anti-GQ1b-positive MFS whole sera, diluted 1:2 in Ringer medium (43,53). The sera induced a dramatic increase in the frequency (up to 300-fold at some NMJs) of spontaneously released ACh quanta, measured as MEPPs, without altering their amplitudes (Fig. 3A). This was followed by block of evoked ACh release resulting in paralysis of the preparation. The effects were very similar to those of the paralytic neurotoxin α-latrotoxin (αLTx). Incubation with MFS total IgG alone was without effect but subsequent treatment with normal serum readily induced them, suggesting the involvement of complement in the phenomenon, as discussed below. Further subclass purification of IgG from two different MFS patients showed that αLTx-like activity co-eluted with anti-GQ1b activity in the same IgG subclass (IgG_3 in one serum studied and IgG_1 in the other), suggesting that these antibodies were the responsible factors. Further support for this hypothesis came from the observation that a cloned human anti-GQ1b IgM, derived from a patient with a chronic IgM paraproteinaemic neuropathy resembling MFS, had similar αLTx-like activity (28,43).

We observed that, upon incubation with MFS sera, fibers of the diaphragm preparations started twitching spontaneously in an asynchronous way. Such a phenomenon is also observed upon incubation with αLTx. The twitches ceased when *d*-tubocurarine, a blocker of the postsynaptic AChR, was added, indicating that the twitches were not caused by malfunction of a process in the synaptic transmission downstream of the AChRs, e.g. spontaneous opening of muscle fiber Na^+ channels. Microelectrode measurements at the NMJs of twitching fibers always revealed high frequency MEPPs (>100/s vs. ~0.5/s before the MFS serum). Sometimes, the MEPPs became superimposed and apparently passed the firing threshold since an action potential was triggered which in turn caused the fiber contraction (Fig. 3B). We have made use of this phenomenon in a recently developed bioassay designed to screen large numbers of sera for their potency in inducing the αLTx-like effects (54). In this assay, mouse

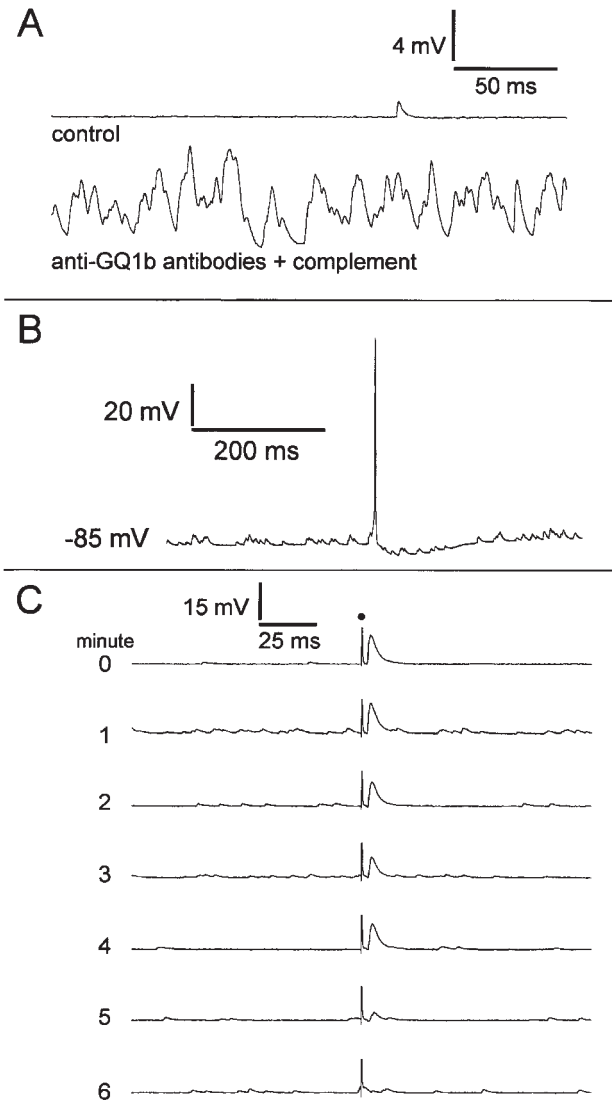

Fig. 3. Electrophysiological effects of anti-GQ1b antibodies on the mouse neuromuscular junction. **(A)** Spontaneous release, measured as the frequency of occurrence of miniature endplate potentials (MEPPs), is about 0.5 ACh quanta per second in the diaphragm neuromuscular junction (NMJ) of young mice at 20°C ex vivo (**upper trace**). When a nerve-muscle preparation has been exposed to anti-GQ1b antibodies (either mouse monoclonals or from Miller Fisher syndrome serum) and human complement is added, the MEPP frequency rises dramatically, sometimes up to values of several hundreds per second (**lower trace**). Such a high level is sustained for 20–30 mins. Thereafter, the MEPP frequency rapidly decays and becomes zero. **(B)** Sometimes, the MEPP frequency becomes so high during the anti-GQ1b antibody/complement treatment that MEPPs become superimposed, pass the firing threshold, and trigger an action potential in the muscle fiber. This explains the occurrence of spontaneous asynchronous fiber twitches in the preparation during the treatment. **(C)** At NMJs where the MEPP frequency becomes high, the ACh release evoked by a nerve action potential becomes gradually blocked, which can be observed as a reduction to zero of the amplitude of the endplate potential (EPP). A black dot indicates the moment of nerve stimulation which can be seen as an artefact on the voltage signal. Traces are the recordings made every minute for a period of 6 min during the incubation period with human complement serum. The preparation had been pre-incubated with a mouse monoclonal anti-GQ1b IgM antibody.

diaphragm strips were incubated with GBS/MFS sera in small volume incubation wells, and the occurrence of twitching was scored. It appeared that 81% (13/16) of the tested anti-GQ1b–positive MFS sera had the αLTx-like effect. For the GBS sera this was 10% (5/50), of which 80% (4/5) were anti-GQ1b positive. Taken together, these data again indicate that anti-GQ1b antibodies are the serum factor responsible for inducing αLTx-like effects at mouse NMJs, although since one anti-GQ1b–negative GBS serum clearly induced αLTx-like effects, other antibodies with similar action may exist.

We confirmed the complement-dependent electrophysiological effects of anti-GQ1b–positive MFS/GBS sera in experiments using anti-GQ1b IgM mAbs that were derived from mice immunized with lipopolysaccharides containing GD3/GT1a-like structures and which originated from MFS/GBS-associated *C. jejuni* strains (38). Besides indicating that molecular mimicry is a likely mechanism in GBS/MFS, these studies provided us with potent anti-GQ1b mAbs (with cross-reactivity to GT1a and GD3) to further characterize the αLTx-like effects.

In a further study we investigated whether the block of evoked ACh release, observed as block of EPPs (Fig. 3C), occurred as a primary effect or whether it appeared secondary to the complement-dependent increase in spontaneous uniquantal ACh release (55). To this end, we measured EPPs and MEPPs at NMJs before and after incubation with a MFS anti-GQ1b-positive IgG or the mouse anti-GQ1b/GD3 mAb CGM3 and calculated the quantal content from their amplitudes. As a positive control we used ωAgatoxin-IVA, which blocks P-type Ca^{2+} channels and thus reduces the quantal content. Anti-GQ1b antibodies alone, i.e., without added complement, did not influence the quantal content, leading us to conclude that block of evoked ACh release is not a primary effect of antibody binding per se, but that it either occurs as a complement-dependent primary effect, in parallel to the induction of high frequency MEPPs, or secondary to the extremely high level of spontaneous ACh release induced by complement activation. In the case of the latter, spontaneous release might result in block of evoked release either by depleting the transmitter store or by causing presynaptic damage, e.g., due to the large amount of membrane incorporation resulting from massive exocytosis. Our observations do not support the first possibility since high frequency MEPPs remain present for some time after the block of evoked ACh release. Presynaptic destruction seems a more likely cause and is supported by ultrastructural observations at CGM3/complement treated mouse NMJs (see below).

On motor nerve terminals, the primary target of anti-GQ1b antibodies mediating the αLTx-like effect could be either GQ1b, a closely related ganglioside, or an unrelated (sialylated) antigen, for instance the αLTx receptor latrophilin (56). However, using mutant mice lacking complex gangliosides (including GQ1b) and a specific anti-GQ1b mAb, we have proven that GQ1b is indeed the primary antigenic target (Bullens et al., unpublished data).

Deposition of Immune Mediators at the Nerve Terminal

In conjunction with the electrophysiological observations described above, we have conducted parallel studies on the immunohistological and pathological sequelae of anti-GQ1b antibody exposure to the hemidiaphragm preparation. Immunohistological analyses of hemidiaphragms exposed to purified IgG from MFS sera and to the anti-GQ1b/GD3 mAb CGM3 have shown that antibody is extensively deposited over the NMJ (Fig. 4). This is consistent with our findings that anti-GQ1b antibodies bind strongly to the NMJ in topical immunostaining studies (28,38,43,55). However, in the tissue from this ex vivo preparation, we found no evidence of antibody penetration and binding to intramuscular nerve-bundles, despite reactive antigens being present in our topical immunostaining studies of muscle and nerve sections (57). It is thus likely that antibody access to nerve fibers and nodes of Ranvier outwith the NMJ is restricted by the blood-nerve barrier in this acute ex vivo preparation, as we have previously observed for isolated sheathed sciatic nerve preparations (58).

Using confocal microscopy, we can partially resolve the presynaptic neuronal elements, the perisynaptic Schwann cell (pSC) and the postsynaptic structures that comprise the NMJ, and the localization of antibody deposits at the NMJ has been examined. As shown in Figure 4, extensive mAb deposits are present on the pSC, as the labelling pattern extends around the nuclei of these cells and co-locates with the Schwann cell specific marker, S100 in double staining studies (not shown). The juxtaposition of mAb deposits to the postsynaptic ACh receptors (as defined by α-bungarotoxin staining) is suggestive of a presynaptic rather than the postsynaptic localisation. We are currently resolving this further by immunogold electron microscopy.

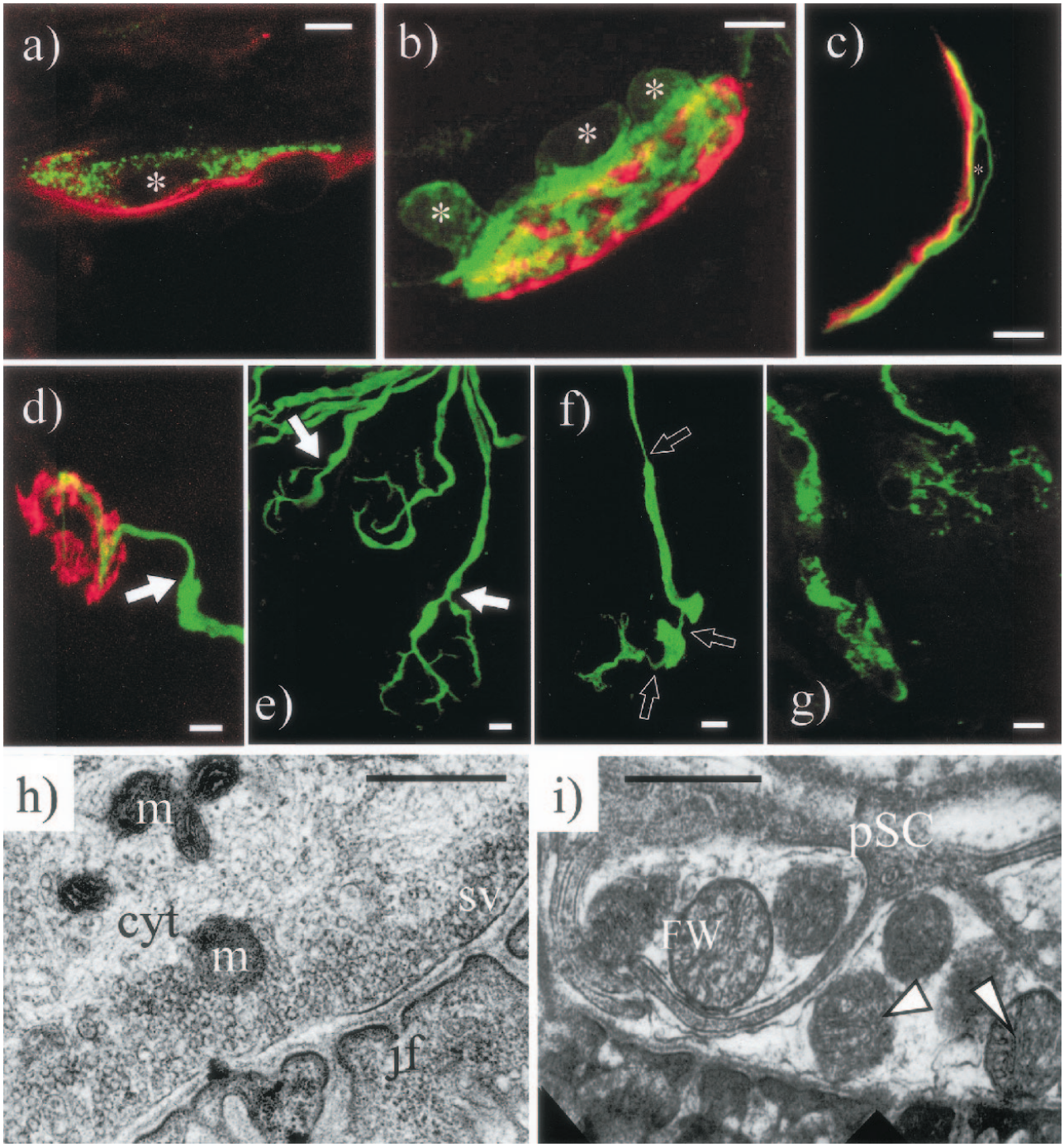

Fig. 4. Microscopic analysis of the αLTx-like lesion. **(a)** Following incubation of the hemidiaphragm preparation with anti-GQ1b mAbs and a source of complement, the immunoglobulin (green) forms punctate deposits over the presynaptic apparatus. The postsynaptic acetylcholine receptors are labelled using α-bungarotoxin (BTx, red). The unstained areas (*) within the presynaptic area probably represent the cytoplasmic spaces of perisynaptic Schwann cells (pSC). **(b and c)** In similar tissue, deposits of complement activation product C3c (green) are localized over the neuromuscular junction as delineated by BTx staining (red). Although staining co-localizes at the synaptic cleft, areas of stain extend away from junctional areas, and again feature pSC cytoplasmic spaces (*). **(d and e)** In normal control junctions the neurofilament (NF) cytoskeleton (green) extends processes over the junctional region (BTx; red). The transition between axon and junction is sometimes marked by a reduction in axon caliber or a constriction (*arrows*). **(f and g)** In muscle preparations pre-incubated with anti-GQ1b antibodies and a source of complement there is evidence of cytoskeletal change. At many endplates the NF of the preterminal axon and junction show marked swellings and constrictions (f), whilst in others (g) the cytoskeleton is fragmented. **(a–g)** All scale bars = 5 μm. **(h)** Electron micrograph of a normal mouse neuromuscular junction. The synaptic cleft, running bottom left to top right, separates the presynaptic apparatus from the postsynaptic junctional folds (jf). Synaptic vesicles (sv) are densely packed close to the presynaptic membrane, beyond which lie mitochondria (m) and cytoskeletal bundles (cyt) (bar = 500 nm). **(i)** In tissue subjected to the αLTx-like lesion, the morphology in greatly disrupted. The terminal is almost completely depleted of synaptic vesicles, and mitochondria are swollen and slumped against the presynaptic membrane (*arrows*). Processes from pSC are inserted into the terminal, and have completely enclosed a portion of the terminal in a "full wrap" (FW). Cytoskeletal bundles are absent (bar = 600 nm).

Activation of complement pathways leads to the generation of the lytic membrane attack complex $C_{5b\text{-}9}$ (MAC). Along with this pore-forming complex, other biologically active products are generated, including the anaphylatoxins C3a, C4a, and C5a. In the hemidiaphragm preparation exposed to the mAb CGM3, in the presence of a complement source provided by normal human serum, complement products are deposited at the NMJ in a very similar distribution to the mAb. Complement fragment C1q, C3c, C5, and MAC can all be detected (Halstead and O'Hanlon, unpublished results). In a series of experiments using complement deficient sera in the hemidiaphragm preparation, the αLTx-like effects did not occur with C5 deficient serum, but continued to occur with C8 deficient serum, suggesting that although it occurs, MAC formation may not be essential for the development of the αLTx-like effect (43). An important intermediate complement component in the development of this lesion might therefore be the anaphylotoxin C5a. Receptors for both C3a and C5a are found in the rodent nervous system on both neuronal and glial cells (O'Hanlon, unpublished observations) (59–61), although their function remains unclear. In our experiments using C8 deficient serum, it is possible that the presence of small amounts of endogenous murine C8 within the nerve-muscle preparation allowed low level MAC formation to occur, thus confounding our interpretation of the role of MAC. Experiments combining hemidiaphragm preparations dissected from C6 deficient mice, subsequently exposed to C6 deficient sera as the complement source, will resolve this issue since in this situation, progression of the complement cascade to MAC formation is not at all possible.

Morphological Changes Occurring at the Nerve Terminal

The axon structure is supported by a framework of neuron-specific cytoskeletal proteins, including neurofilament (NF) and β-tubulin, which extend into the presynaptic nerve terminal overlying the post-synaptic apparatus. Using a combination of quantitative immunohistological and image analysis techniques, we have shown that in tissue exposed to human or murine anti-GQ1b antibodies and a source of complement, the signal for both the NF and β-tubulin proteins is severely depleted when compared to control tissue, and have interpreted these findings as reflecting the destruction of the NMJ cytoskeleton (62). In further quantitative studies, we have shown that there is an inverse correlation between the average size of C3c complement deposits at the NMJ, and the corresponding NF signal, thereby strongly supporting our view that the NF-loss is dependent upon the degree of complement activation (O'Hanlon, unpublished results).

Ultrastructural analysis of NMJ from hemidiaphragm tissue exposed to anti-GQ1b/GD3 antibodies and complement demonstrates severe destructive changes that reinforce and extend our immunohistological observation of NMJ cytoskeletal loss. In view of the variations in normal NMJ ultrastructural appearance, we have quantified a range of morphological parameters at the electron microscopic (EM) level that are significantly different from control tissue. In CGM3/complement treated tissues, the vast majority of nerve terminals show widespread abnormalities, including displaced, swollen, and damaged mitochondria and a reduced contact with the associated underlying muscle. Many nerve terminals had pSC processes intruding into the synaptic cleft, and in some cases the nerve terminal appeared to be divided into smaller units by invading pSC processes, some of which form a "full wrap," completely encasing and isolating a portion of the neuronal element of the nerve terminal (62).

Insights into the Mechanism of Nerve Terminal Injury

Our working hypothesis for the mechanisms underpinning the αLTx-like effects of anti-GQ1b/GD3 antibodies and complement is summarised in Figure 5. It is clear from our studies that the anti-GQ1b antibody mediated lesion we have observed is dependent upon complement activation at the NMJ. One likely effect of complement activation is increased intracellular $[Ca^{2+}]$ in the nerve terminal, either occurring directly via Ca^{2+} influx through MAC pores, or indirectly via a complement receptor mediated pathway that induces influx of external Ca^{2+} or release of Ca^{2+} from internal stores. The rise in cytosolic $[Ca^{2+}]$ would then directly induce quantal release, leading to increased MEPP frequency. Additionally, MAC pore formation is likely to lead to an inability to maintain the nerve-terminal resting membrane potential through unregulated cation fluxes, resulting in the opening of voltage gated Ca^{2+} channels, as would normally occur in response to a nerve action potential. It is already recognised that the pores formed by αLTx cause nerve terminal depolarisation and result in the retrograde propagation of nerve action potentials (63).

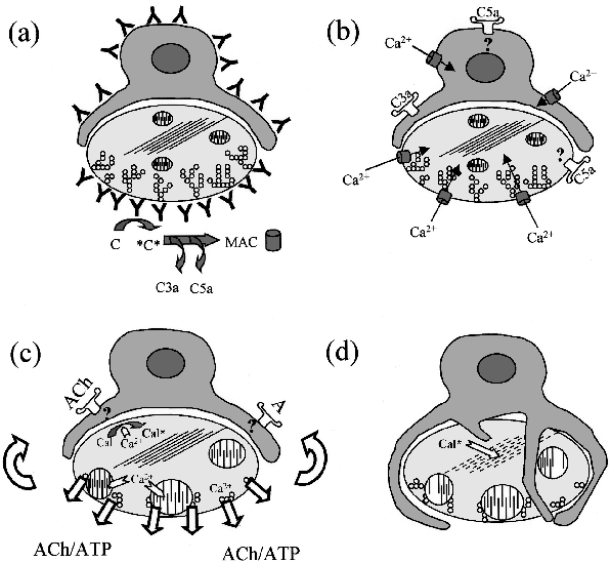

Fig. 5. Schematic representation of the αLTx-like effects of anti-GQ1b/GD3 antibodies and complement on the mouse neuromuscular junction. **(a)** Anti-GQ1b/GD3 mAb deposits (Y) suggest the presence of reactive epitopes on both nerve terminal and perisynaptic Schwann cells (pSC). There is no evidence of post-synaptic binding in this model, thus for clarity the muscle side of the synapse is not shown here. Binding of anti-GQ1b antibody alone has little or no effect, either electrophysiologically or morphologically. In the additional presence of normal human serum as a complement source, the complement cascade is triggered. **(b)** Complement deposits co-locate with antibody deposits, and we have identified C1q, C3c, C5 and the pore forming membrane attack complex (MAC, $C_{5b\text{-}9}$). The deposits of the latter are likely to facilitate Ca^{2+} ingress into both the nerve terminal and pSC. Additionally, receptors for the complement anaphylotoxins, C3a and C5a, are present on both nerve and pSC, although it is unclear what signalling events may be triggered by receptor-ligand binding. **(c)** Calcium ingress into the nerve terminal causes a massive release of acetylcholine (ACh) and co-release of ATP. Receptors for both adenosine (A) and ACh are present on the pSC, and are thought to be important components of the signalling pathways that exist between these cells and the underlying nerve terminal. Their role in mediating pathological changes is uncertain. The nerve terminal becomes swollen, probably though a combination of synaptic vesicle membrane addition, and the osmotic effects brought about by MAC pore formation. The final physiological result is that neuromuscular transmission becomes blocked due to failure of evoked ACh release, which leads to paralysis. **(d)** Activated calpain destroys the cytoskeleton of the nerve terminal. Having been triggered to adopt a phagocytic phenotype, pSC insert processes into the synaptic cleft and through the nerve terminal, ultimately completely enfolding portions of the nerve terminal.

At the mammalian NMJ, pSCs respond metabolically to the release of ACh and co-released ATP through the activation of muscarinic and adenosine A1 receptors (64,65). The physiological consequences of anti-GQ1b antibody–induced, large scale, uncontrolled transmitter release on pSCs are not known, but this may contribute to the development of an activated, phagocytic pSC phenotype.

As well as triggering exocytosis, an uncontrolled rise in intraterminal [Ca^{2+}] would also trigger other Ca^{2+}-dependent processes. It is likely that loss of cytoskeletal proteins is due to the action of Ca^{2+}-activated neutral cysteine proteases termed calpains, that have been implicated in many aspects of neural development, neurodegenerative change, and apoptotic cell death (66–68). There are two major calpain isoforms, μ (I) and m (II), named from the molarity of Ca^{2+} (μM and mM, respectively) required to activate them, and both isotypes may be important in degenerative conditions of the peripheral motor system (69–72). It has been hypothesized that loss of the filamentous structure of NF only occurs when intra-axonal calcium levels allow activation of m calpain. Below this level, NF compaction occurs due to sidearm cleavage (73). When analysing NF content by quantitative immunohistological analysis, one might therefore expect to find an intensification of the NF signal in a mild lesion occurring at submillimolar elevations in [Ca^{2+}], and a loss of NF signal at high [Ca^{2+}]. This prediction is supported by our finding of an increased NF signal in preparations treated with human or mouse anti-GQ1b antibodies in the absence of a heterologous complement source, in which we have shown a low level activation of endogenous mouse complement occurs (O'Hanlon et al., unpublished results).

Under physiological circumstances, Ca^{2+} enters the nerve terminal through voltage gated Ca^{2+} channels upon the arrival of a nerve action potential and is quickly removed from the cytosol, either by sequestration into internal storage vesicles or by removal from the cell by Ca^{2+}-ATPase and the Na^{+}/Ca^{2+} exchanger. Additionally at high [Ca^{2+}], mitochondria are also able to sequester cytosolic Ca^{2+}, although their ability to do so is temperature dependent and may therefore be compromised in the hemidiaphragm preparation maintained at room temperature (74). In both αLTx treated tissue (75) and in our anti-GQ1b/complement model system, the mitochondrial swelling we see may be a result of a high intra-terminal [Ca^{2+}] and/or of the intense metabolic demands of an increased rate of exocytosis and Ca^{2+} homeostatic processes in response to Ca^{2+} overload. In addition to swelling, the mitochondria tend to slump onto the presynaptic membrane which may be due simply to the removal of intervening synaptic vesicles, or the loss of a cytoskeletal matrix, or a combination of both.

In view of the substantial antibody and complement deposits observed on pSCs, the possibility exists that the immune attack is focused primarily on the pSC, and all other events, including neuronal exocyto-

sis, occur as a consequence of pSC activation. It is likely that the pSC also undergoes a rise in intracellular [Ca^{2+}]. Whether these changes in [Ca^{2+}] cause a lethal pSC injury is unknown, but this seems unlikely since many pSCs subsequently take on a phagocytic role, engulfing the nerve terminal. With respect to myelinating SCs, complement activation has been shown to cause demyelination without apparently affecting SC survival in vitro (76). Additionally, sublytic complement activation in cultured SCs has been shown to trigger changes in gene expression, and stimulates mitogenesis and apoptotic rescue (77,78). The pathophysiological relevance of these in vitro observations remain unclear, but such signals may contribute to the transformation of the pSC from a support role for the underlying neuron, to a phagocytic phenotype.

Converting the *ex Vivo* Hemidiaphragm Preparation into an In Vivo Model of MFS

Mice passively immunized with mouse anti-GQ1b mAbs remain clinically normal and lack any obvious morphological or electrophysiological features of nerve terminal injury (38). Passively immunized mice do nevertheless have low level deposits of both immunoglobulin and complement products at the NMJ, indicating that anti-GQ1b antibodies can target this site in vivo, and fix small amounts of complement. Furthermore, in ex vivo nerve-muscle tissues dissected from these passively immunized mice, heterologous (human) complement readily produces the known αLTx-like effects at NMJs. There are several reasons why the mouse may be relatively resistant to in vivo effects, yet vulnerable to in vitro exposure to anti-GQ1b antibody and complement. Firstly, all passive immunization studies have so far been performed using IgM anti-GQ1b mAbs. In these animals, serial analysis of mouse serum samples and NMJs for anti-GQ1b IgM activity and IgM deposits respectively have shown that IgM mAb is rapidly cleared from the mouse circulation and only evident as IgM deposits at nerve terminals in the first 24–48 hours following passive immunization. Since IgG has a longer circulating half-life, we are currently developing passive immunization protocols using complement fixing IgG mAbs that should help to overcome this factor.

A second issue concerns the activity of mouse complement and complement regulatory proteins, as alluded to above. In the ex vivo preparation, mouse complement is unable to provide the required complement environment for the αLTx-like effects to develop, and we thus supplement the preparation with normal human serum as a source of complement which is not only highly active, but also heterologous (i.e., non-mouse). This helps to circumvent the complement regulatory mechanisms that would normally override self-injury arising from tissue deposition of mouse complement. The mouse has a relatively inactive complement system in comparison with species such as the rabbit or rat and as such is not ideal for modelling antibody-mediated autoimmune disease. However, other factors, such as its small size and the ready availability of genetically modified animals make the mouse the most preferable species in which to model MFS.

CONCLUSIONS

Despite considerable gaps in our knowledge, Miller Fisher syndrome remains the best understood of the acute inflammatory neuropathies in terms of the overall pathogenic cycle. It is evident that complement fixing IgG anti-GQ1b antibodies can arise through molecular mimicry with microbial oligosaccharides as part of a primary immune response to the triggering infection. The anti-GQ1b antibodies then circulate into the extracellular compartments where they have free access to bind selectively to GQ1b-enriched sites in the nervous system, particularly those that are not well protected by the blood nerve barrier. These sites most likely include motor nerve terminals innervating extraocular muscles, muscle spindles and structures in the dorsal root ganglia, and this accounts for the unique clinical features seen in the syndrome. Once bound to neural membranes, anti-GQ1b antibodies initiate a complement dependent inflammatory injury. As the primary immune response decays, the clinical deficit recovers spontaneously, provided irreversible neural injury has not occurred. Although simplistic, this pathway sets out a framework by which immunological and pathophysiological mechanisms can be explored and treatment options evaluated. What remains to be established is the clinical relevance of nerve terminal injury, as opposed to segmental demyelination of extraocular nerves, and this requires further exploration through more detailed neurophysiological studies in man. Our experimental observations laid out in this review strongly indicate that such studies are warranted.

Irrespective of the involvement of the nerve terminal as a site for anti-GQ1b antibody–mediated injury, it seems inconceivable that anti-GQ1b antibodies are not the primary causal agent in MFS, and as such the syn-

drome has the capacity to act as a paradigm on which to build a vast wealth of equivalent data for other anti-glycosphingolipd antibody specificities and their associated clinical syndromes.

ACKNOWLEDGMENTS

The work of the authors that is described in this review was supported by the Prinses Beatrix Fonds (R. W. M. B., #96-0210), KNAW Van Leersumfonds (J. J. P.), the Stichting 'De Drie Lichten' (R. W. M. B.), The Wellcome Trust (G. M. O., H. J. W.), and the Guillain Barré Syndrome Support Group, UK (G. M. O., H. J. W.).

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