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An animal model of Miller Fisher syndrome: Mitochondrial hydrogen peroxide is produced by the autoimmune attack of nerve terminals and activates Schwann cells

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ABSTRACT

The neuromuscular junction is a tripartite synapse composed of the presynaptic nerve terminal, the muscle and perisynaptic Schwann cells. Its functionality is essential for the execution of body movements and is compromised in a number of disorders, including Miller Fisher syndrome, a variant of Guillain-Barré syndrome: this autoimmune peripheral neuropathy is triggered by autoantibodies specific for the polysialogangliosides GQ1b and GT1a present in motor axon terminals, including those innervating ocular muscles, and in sensory neurons. Their binding to the presynaptic membrane activates the complement cascade, leading to a nerve degeneration that resembles that caused by some animal presynaptic neurotoxins.

Here we have studied the intra- and inter-cellular signaling triggered by the binding and complement activation of a mouse monoclonal anti-GQ1b/GT1a antibody to primary cultures of spinal cord motor neurons and cerebellar granular neurons. We found that a membrane attack complex is rapidly assembled following antibody binding, leading to calcium accumulation, which affects mitochondrial functionality. Consequently, using fluorescent probes specific for mitochondrial hydrogen peroxide, we found that this reactive oxygen species is rapidly produced by mitochondria of damaged neurons, and that it triggers the activation of the MAP kinase pathway in Schwann cells.

These results throw light on the molecular and cellular pathogenesis of Miller Fisher syndrome, and may well be relevant to other pathologies of the motor axon terminals, including some subtypes of the Guillain Barré syndrome.

1. Introduction

Miller Fisher syndrome (MFS) is a peripheral neuropathy characterized by ophthalmoplegia, ataxia, and areflexia (Fisher, 1956). The pathogenic factors are autoantibodies specific for the oligosaccharide portion of specific polysialogangliosides, mainly GQ1b and GT1a (Chiba et al., 1993). These antibodies form during the immune response against some viral or bacterial infections, and bind microbial antigens that mimic nerve polysialogangliosides, highly enriched in murine nerve terminals in the diaphragm (Yuki et al., 1994; Goodyear et al., 1999). Anti-GQ1b/GT1a autoantibodies are also associated with incomplete forms of MFS, including an acute ophthalmoparesis without ataxia, an acute ataxic neuropathy without ophthalmoplegia, and its central nervous system subtype termed Bickerstaff brain-stem encephalitis (Wakerley et al., 2014). All these syndromes benefit from plasmapheresis and intravenous immunoglobulin administration and are reversible.

The common pathogenic step is the binding of autoantibodies to presynaptic neuronal membranes enriched in the polysialogangliosides GQ1b and GT1a, and the ensuing recruitment of complement (O'Hanlon et al., 2001). The activated complement forms a transmembrane pore named membrane attack complex (MAC), allowing the rapid entry of Ca^{2+} which triggers degeneration of the axon terminals (Orrenius et al., 2003; Bano and Nicotera, 2007; Rupp et al., 2012). This form of neurodegeneration appears to be confined to the axon terminal of peripheral neurons.

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In many respects this autoimmune pathology resembles the one caused by animal presynaptic neurotoxins, which cause a rapid uptake of Ca^{2+} ions within the motor axon terminal. Experiments performed in *ex-vivo* murine nerve-muscle preparations show that the antibody *plus* complement complex bound to the presynaptic membrane damages the neuromuscular junction (NMJ) very similarly both morphologically and electrophysiologically to α-latrotoxin (Halstead et al., 2004), with paralysis and degeneration of nerve endings. In mice the disease is very similar to the human pathology with reversible paralysis of the NMJ, but with a more rapid time course. Complete regeneration is usually achieved within five days, as it occurs following α-latrotoxin poisoning (Duregotti et al., 2015).

The molecular and cellular events involved in the reversible degeneration of motor axon terminals by anti-ganglioside antibodies *plus* complement are ill-known, and are the focus of the present study. Here, we have used a mouse monoclonal anti-GQ1b/GT1a antibody, previously characterized as the MFS inducer (Koga et al., 2005), and have studied the intra- and intercellular signaling events triggered by the anti-ganglioside antibody *plus* complement complex at the murine NMJ, in two types of primary cultured neurons, and in co-cultures of neurons with Schwann cells (SCs).

2. Materials & methods

2.1. Chemicals

The mouse monoclonal antibody (FS3, isotype IgG2b-κ) was previously characterized (Koga et al., 2005). For immunization mice were inoculated with a heat-killed *C. jejuni* lysate, the infectious agent frequently associated with MFS. FS3 recognizes gangliosides GQ1b and GT1a, the latter being identical to GQ1b except for one sialic acid residue less. Normal human serum (NHS) from a pool of human healthy males AB plasma (Sigma-Aldrich #H4522, lot #SLBG2952V) was employed as a source of complement. Unless otherwise stated, all reagents were purchased from Sigma.

2.2. Mice

Experiments were performed in Swiss-Webster adult male CD1 mice. All procedures were performed in accordance with the Council Directive 2010/63/EU of the European Parliament and approved by the Italian Ministry of Health.

2.3. NMJ immunohistochemistry

For binding studies whole LAL and EOMs were incubated *ex-vivo* with FS3 10 μg/mL at 10 °C for 15–30 min, then washed, fixed and processed for immunofluorescence (see below).

For MAC deposition analysis FS3 (10 μg) was diluted with NHS 50% (v/v) in 100 μL of physiological saline (0.9% wt/v NaCl in distilled water), and injected s.c. in proximity of LAL muscle of anesthetized CD1 of around 20–25 g; muscles were collected after 2 h. In the case of EOMs, an *ex vivo* incubation was performed (FS3 10 μg/mL + NHS 50% v/v, 1 h at 37 °C). Heat inactivation of NHS (56 °C for 30 min, HI-NHS), or treatment with NHS 50% alone were employed as negative controls.

To define the kinetics of nerve terminal degeneration and regeneration in mice, FS3 (10 μg) was diluted with NHS 50% (v/v) in 100 μL physiological solution, and subcutaneously injected close to LAL muscles, or intramuscularly in the mice hind limb for different time points. Muscles were then fixed in 4% (wt/v) PFA in PBS for 15 min at room temperature, quenched in PBS + 50 mM NH_4Cl, and then permeabilized and saturated in blocking solution: 15% (v/v) goat serum, 2% (wt/v) BSA, 0.25% gelatin, 0.20% (wt/v) glycine, and 0.5% Triton X-100 in PBS 2 h at room temperature. Incubation with the following primary antibodies was carried out for 48–72 h in blocking solution: anti-neurofilaments (mouse monoclonal, anti-NF200, 1:200, Sigma), anti-VAchT (rabbit polyclonal, 1:1000, Synaptic Systems), anti-C5b-9 (rabbit polyclonal, 1:1000, Abcam). Muscles were then washed and incubated with secondary antibodies (Alexa-conjugated, 1:200, Life Technologies). Nuclei were stained with Hoechst. NMJs were identified by Alexa-conjugated α-bungarotoxin (α-BTx). Images were collected with a Leica SP5 confocal microscope equipped with a 63× HCX PL APO NA 1.4.

2.4. Electrophysiological recordings

Mice were sacrificed at scheduled times by anaesthetic overdose followed by cervical dislocation, soleus muscles dissected and subjected to electrophysiological measurements. Three mice were used for each condition at each time point. Electrophysiological recordings were performed in oxygenated Krebs-Ringer solution on sham or FS3 + NHS injected soleus muscles using intracellular glass microelectrodes (WPI, Germany) filled with one part of 3 M KCl and two parts of 3 M CH_3COOK.

Evoked neurotransmitter release was recorded in current-clamp mode, and resting membrane potential was adjusted with current injection to −70 mV. Evoked junction potentials (EJPs) were elicited by supramaximal nerve stimulation at 0.5 Hz using a suction microelectrode connected to a S88 stimulator (Grass, USA). To prevent muscle contraction, samples were incubated for 10 min with 1 μM μ-Conotoxin GIIIB (Alomone, Israel). Signals were amplified with intracellular bridge mode amplifier (BA-01×, NPI, Germany), sampled using a digital interface (NI PCI-6221, National Instruments, USA) and recorded by means of electrophysiological software (WinEDR, Strathclyde University). EJPs measurements were carried out with Clampfit software (Molecular Devices, USA), statistical analysis with Prism (GraphPad Software, USA).

2.5. Primary cell cultures and co-cultures

Rat cerebellar granular neurons (CGNs), spinal cord motor neurons (SCMNs), primary SCs and their relative co-cultures were prepared as described previously (Rigoni et al., 2007; Duregotti et al., 2015).

2.6. Immunofluorescence

For binding experiments CGNs (6 DIV, *days in culture*) or SCMNs (4–5 DIV), plated onto 35-mm dishes or 24 well-plates, were exposed to FS3 0.1 μg/mL at 16 °C for 20 min in Krebs Ringer buffer for CGNs (KRH: Hepes 25 mM at pH 7.4, NaCl 124 mM, KCl 5 mM, $MgSO_4$ 1.25 mM, $CaCl_2$ 1.25 mM, KH_2PO_4 1.25 mM, glucose 8 mM), and E4 medium for SCMNs (E4: 120 mM NaCl, 3 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, and 10 mM Hepes, pH 7.4). Cells then washed, and subjected to immunofluorescence (see below).

For studies on MAC deposition and *bulge* characterization neurons were exposed to FS3 0.1 μg/mL + NHS 0.5% (v/v) at 37 °C for 20 min.

Following treatments cells were washed, fixed for 10 min in 4% (wt/v) paraformaldehyde (PFA) in PBS and quenched (0.38% glycine, 50 mM NH_4Cl in PBS) at room temperature. Cells were permeabilized and saturated in buffer A (20 mM PIPES, 137 mM NaCl, 2.7 mM KCl, pH 6.8) containing 5% (v/v) goat serum, 50 mM NH_4Cl and 0.5% (wt/v) saponin for 45 min, followed by an overnight incubation in buffer A *plus* 5% goat serum and 0.1% (wt/v) saponin with anti-C5b-9 (rabbit polyclonal, 1:5000, Abcam), or VAMP2 (1:500, Rossetto et al., 1996) primary antibodies. After washes, samples were incubated with the correspondent secondary antibodies (Alexa-conjugated, 1:200; Life Technologies) in buffer A *plus* 5% goat serum and 0.1% (wt/v) saponin for 45 min and washed in buffer A. Nuclei were stained with Hoechst. Coverslips were mounted in ProLong Diamond (Thermo Fisher) and examined by epifluorescence (Leica CTR6000) microscopy.