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The intrinsic pathogenic role of autoantibodies to aquaporin 4 mediating spinal cord disease in a rat passive-transfer model

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ABSTRACT

Neuromyelitis optica (NMO) is causally linked to autoantibodies (ABs) against aquaporin 4 (AQP4). Here, we focused on the pathogenic effects exclusively mediated by human ABs to AQP4 *in vivo*. We performed cell-free intrathecal (i.th.) passive transfer experiments in Lewis rats using purified patient NMO immunoglobulin G (IgG) and various recombinant human anti-AQP4 IgG-ABs via implanted i.th. catheters. Repetitive application of patient NMO IgG fractions and of recombinant human anti-AQP4 ABs induced signs of spinal cord disease. Magnetic resonance imaging (MRI) revealed longitudinal spinal cord lesions at the site of application of anti-AQP4 IgG. Somatosensory evoked potential amplitudes were reduced in symptomatic animals corroborating the observed functional impairment. Spinal cord histology showed specific IgG deposition in the grey and white matter in the affected areas. We did not find inflammatory cell infiltration nor activation of complement in spinal cord areas of immunoglobulin deposition. Moreover, destructive lesions showing axon or myelin damage and loss of astrocytes and oligodendrocytes were all absent. Immunoreactivity to AQP4 and to the excitatory amino acid transporter 2 (EAAT2) was markedly reduced whereas immunoreactivity to the astrocytic marker glial fibrillary acid protein (GFAP) was preserved. The expression of the NMDA-receptor NR1 subunit was down-regulated in areas of IgG deposition possibly induced by sustained glutamatergic overexcitation. Disease signs and histopathology were reversible within weeks after stopping injections. We conclude that *in vivo* application of ABs directed at AQP 4 can induce a reversible spinal cord disease in recipient rats by inducing distinct histopathological abnormalities. These findings may be the experimental correlate of "penumbra-like" lesions recently reported in NMO patients adjacent to effector-mediated tissue damage.

Introduction

The discovery of aquaporin 4 (AQP4) as the target antigen for IgG autoantibodies (ABs) in neuromyelitis optica (NMO) was a significant advancement in our understanding of central nervous system (CNS) autoimmune disorders (Lennon et al., 2004, 2005). Beside tissue destruction with complement deposition and macrophage infiltrates, NMO lesions show extensive loss of AQP4 pointing to an AB-mediated, target-specific humoral immune reaction with a host of proinflammatory components (Misu et al., 2007; Roemer et al., 2007).

Previous *in vivo* studies have provided evidence for a pathogenic role of ABs against AQP4 in a co-existing inflammatory milieu (Bennett et al., 2009; Bradl et al., 2009). Moreover, injection of anti-AQP4 ABs into the brain, incubation of spinal cord slice cultures, or primary astrocytes induced AQP4 depletion and death of astrocytes when activated human complement was present (Ratelade et al., 2012; Saadoun et al., 2010; Wrzos et al., 2014). These observations point to cooperative immunopathological events involving anti-AQP4 ABs, complement, and cell-dependent factors as the cause of tissue destruction in NMO.

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In AB-mediated neurological diseases of the peripheral and central nervous system, ABs typically have intrinsic effects upon binding to their target antigen e.g. in myasthenia gravis (Toyka et al., 1977), in Lambert–Eaton myasthenic syndrome (Buchwald et al., 2005; Fukunaga et al., 1983), autoimmune encephalitis (Hughes et al., 2010), and stiff-person syndrome (Geis et al., 2010). There is an ongoing debate whether ABs against AQP4 in principal might also have pathogenic effects that are independent from complement activation and secondary cell-mediated inflammatory activity. At the cellular level, it has been shown that the glial excitatory amino acid transporter EAAT2 was co-internalized along with AQP4 in transfected cells upon exposure to anti-AQP4-ABs. This led to the hypothesis that glutamate excitotoxicity might contribute to CNS pathology in anti-AQP4 AB-associated autoimmunity (Hinson et al., 2008; Marignier et al., 2010; Wrzos et al., 2014). Other investigators could not find evidence supporting these mechanisms in primary cell culture experiments and after single intracerebral injection of ABs to AQP4 (Ratelade et al., 2011; Rossi et al., 2012). An autopsy series of NMO patients showed loss of AQP4 on reactive astrocytes in the superficial cortical layers accompanied by neuronal loss and microglia activation in adjacent layers. The authors suggested a clinically relevant neurodegenerative process that was concurrent with inflammatory demyelination (Blanc et al., 2008; Saji et al., 2013). Very recently, "penumbra-like" lesions with loss of AQP4 but preserved astrocytes have been reported in spinal cord sections in autopsy cases of NMO patients adjacent to destructive lesions with complement activation (Misu et al., 2013) and after single intracerebral injection of anti-AQP4 AB in rats (Asavapanumas et al., 2014).

We here investigated the intrinsic effects of anti-AQP4 IgG ABs *in vivo* in a chronic animal model with repetitive intrathecal (i.th.) application of NMO patient-IgG or recombinant antibodies (rABs) against AQP4 (Geis et al., 2010, 2011). This approach allows testing AB-mediated pathology in the spinal cord in the living rat over several weeks, independent of additional effector mechanisms.

Materials and methods

Patients, therapeutic plasma exchange, and preparation of IgG fractions

All four NMO patients from whom plasma exchange material was available fulfilled the revised diagnostic criteria for NMO (Wingerchuk et al., 2006) and had positive serum titers of ABs against AQP4. Human NMO immunoglobulin G (NMO1-4) was purified from plasma filtrates as described previously (Buchwald et al., 2002) and from disease-control patients with chronic demyelinating inflammatory polyneuropathy who were negative for anti-AQP4 reactivity (control IgG). Purified IgG from one patient (NMO3) was previously utilized and described in another study (Bradl et al., 2009). All IgG fractions were dialyzed against distilled water, freeze dried and stored at −20 °C. Lyophilized IgG was dissolved in normal saline just before use (100 mg/ml concentration for NMO1, 2, and 4, and control IgG 1 and 2; 12 mg/ml for NMO3). Titers of anti-AQP4 ABs were then measured at a 1 mg/ml concentration by a commercial indirect immunofluorescence test with AQP4 transfected HEK 293 cells (Euroimmun Lübeck, Germany). All purified NMO IgG fractions displayed a strongly positive anti-AQP4 binding pattern (titers ≥1:100).

Recombinant patient antibodies

Purified human recombinant IgG ABs were generated as previously described (Bennett et al., 2009). Recombinant antibodies rAB^{AQP4} and rAB^{contr2} were generated from plasmablast clones recovered from a NMO patient's cerebrospinal fluid (CSF). rAB^{contr2} is specific for human AQP4 but does not bind rat AQP4 (Bennett et al., 2009). rAB^{contr1} is an isotype-matched control human recombinant AB specific for measles virus nucleocapsid protein (Bennett et al., 2009).

Animals, surgery, and intrathecal injections

A total of 112 female Lewis rats were included in the experiments reported here. Adult rats (8–10 weeks old) were purchased from Harlan-Winkelmann (Borchen, Germany). All experiments were approved by the Bavarian and Thuringian State authorities. Intrathecal (i.th.) catheters (0.61-mm outer diameter, intrathecal length 6.5 cm) were placed as described (Geis et al., 2010; Yaksh and Rudy, 1976). Rats developing signs of paralysis after surgery with catheter placement were sacrificed immediately. All others were allowed to recover for 8–10 days, and rats were randomized to receive either purified patient IgG or recombinant ABs. The principal experimental groups contained a minimum of 6 animals for each treatment mode. All i.th. injections were painless as judged by daily observation of the rats and were done in the awake rat while gently immobilizing them for 30 seconds.

The IgG fractions were injected at the following concentrations: NMO IgG 1, 2, 4 or control IgG 1 or 2 at 100 mg/ml, NMO IgG 3 at 12 mg/ml; for estimating a dose–response relation, NMO IgG 4 was injected at 30 mg/ml and 70 mg/ml in two groups of 3 rats each; rAB^{AQP4} was injected at a concentration of 1 mg/ml (or of 0.5 mg/ml in 7 rats for estimating the dose–response relation) and rAB^{contr1}, rAB^{contr2} at 1 mg/ml. Because of their monoclonality for the respective antigens, rAB IgG fractions were used in a 100-fold lower concentration than polyclonal NMO-IgG or control IgG fractions. The high concentrations of patient IgG and of control IgG did not produce any unspecific effects as observed in this and in previous experiments (Geis et al., 2010, 2012). Injections of 10 μl purified NMO IgG or rABs or control IgG were performed with a subsequent flush of 10 μl saline in three series of daily injections for 5 days each with a 2 days break before the subsequent series, resulting in 15 total IgG injections over a 3 week period (Fig. 1). This approach was chosen because it had been found useful in earlier studies (Geis et al., 2010, 2011). Moreover, the action of ABs can be studied independent of additional co-factors including complement, and the time course of developing disease signs can be followed. In a further group of animals we studied the recovery potential using the IgG fraction named NMO IgG 3 after a full course of injections observing the rats for another 30 days without any further IgG application.

Behavioral analyses

Behavioral analyses were performed by investigators who were masked as to the treatment assignments. Animals were observed daily for at least one hour while moving in their cages and freely over a large table. Body weight was monitored daily. Motor disability was rated on a spinal cord disease score modified from an EAE score (Linker et al., 2002) ranging from 0 (no symptoms) to 10 (death due to complete immobilization; Supplementary Table). Before surgery, rats were trained on an accelerating RotaRod (TSE Systems, Bad Homburg, Germany) and quantitative testing was performed 8–10 days after surgery just before the 1st injection to be taken as baseline, and then after the 5th, 10th and 15th i.th. injections (Fig. 1). Grip strength of the forelimbs was tested with a digital grip force meter (DFIS series, Chatillon, Greensboro, NC, USA) before and after surgery and after the 5th, 10th and 15th i.th. injection.

Magnetic resonance imaging and contrast agent

The experimental contrast agent gadofluorine M (a gift from Bayer Schering Pharma GmbH, Berlin, Germany), which is known to be up to 10 times more sensitive than standard gadolinium in detecting leakage of the blood–brain barrier, was applied intravenously in the tail vein of experimental rats 24 hours before MRI imaging (0.1 mmol/kg) (Bendszus et al., 2008). All measurements of rat spinal cord were performed in the anesthetized rat on a 3T MRI Unit (Siemens TIM Trio, Erlangen, Germany) using a commercial 7 cm coil (Siemens). Rats were placed in a custom-made plastic chamber in a stretched position.