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Antibodies Produced by Clonally Expanded Plasma Cells in Multiple Sclerosis Cerebrospinal Fluid Cause Demyelination of Spinal Cord Explants

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Abstract

B cells are implicated in the etiology of multiple sclerosis (MS). Intrathecal IgG synthesis, cerebrospinal fluid (CSF) oligoclonal bands and lesional IgG deposition suggest a role for antibody-mediated pathology. We examined the binding of IgG1 monoclonal recombinant antibodies (rAbs) derived from MS patient CSF expanded B cell clones to central nervous system (CNS) tissue. MS rAbs displaying CNS binding to mouse and human CNS tissue were further tested for their ability to induce complement-mediated tissue injury in ex vivo spinal cord explant cultures. The staining of CNS tissue, primary human astrocytes and human neurons revealed a measurable bias in MS rAb binding to antigens preferentially expressed on astrocytes and neurons. MS rAbs that recognize myelin-enriched antigens were rarely detected. Both myelin-specific and some astrocyte/neuronal-specific MS rAbs caused significant myelin loss and astrocyte activation when applied to spinal cord explant cultures in the presence of complement. Overall, the intrathecal B cell response in multiple sclerosis binds to both glial and neuronal targets and produces demyelination in spinal cord explant cultures implicating intrathecal IgG in MS pathogenesis.

Keywords

multiple sclerosis; monoclonal antibody; demyelination; autoimmunity; neuroimmunology; spinal cord slice cultures

INTRODUCTION

Multiple Sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) of unknown cause. Despite extensive investigation and

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characterization of active MS lesions, no consensus regarding a uniform mechanism of disease has emerged, and the possibility of multiple pathogenic pathways has been widely debated [18,14,7]. The efficacy of rituximab, a therapy targeting peripheral CD20+ B cells, in treating relapsing-remitting forms of MS [13] has renewed interest in the role B cells may play in disease: antigen-presentation, pro-inflammatory cytokine secretion, and antibody production [5,2,17]. Because active MS lesions are often characterized by deposition of immunoglobulin G (IgG) and activated complement products in conjunction with macrophage-mediated myelin destruction (3), antibodies could play a direct role in inflammatory CNS injury.

Intrathecal IgG synthesis is one of the most striking biochemical hallmarks of disease [15,31] and is accompanied by elevated numbers of clonally expanded B and plasma cells in MS CSF and brain tissue [10,30,23,1,22]. Because CSF B cell clones produce IgG against relevant infectious agents and autoantigens in a range of human CNS disease [12,20,9,4], we hypothesize that the oligoclonal IgG produced by MS CSF plasmablasts [19] is directed against disease-relevant antigens. Towards a better understanding of this response, we have previously produced human IgG1 monoclonal recombinant antibodies (rAbs) from expanded MS CSF plasmablast clones. [21]. MS CSF-derived rAbs do not recognize the major myelin proteins myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP), or myelin basic protein (MBP) when expressed in tissue culture cells and demonstrate negligible immunoreactivity on formalin-fixed and paraffin embedded MS and control tissues [21]. A subset of MS rAbs, however, shows strong reactivity to myelin-enriched glycolipid complexes spotted onto PDVF membranes, although CSF-derived rAbs from other CNS inflammatory diseases bound to glycolipid complexes with similar frequencies [8].

In this study, we further evaluated MS CSF-derived rAbs for reactivity against antigens expressed in immortalized glial cell lines, primary human astrocytes and neurons, and on paraformaldehyde (PFA)-fixed human and mouse brain tissue sections. We then utilized spinal cord explant cultures to assess the effect of CNS-reactive MS rAbs on intact CNS tissue. Multiple MS rAbs, but none of three isotype control CSF rAbs, promoted myelin damage, astrocyte activation, and complement deposition suggesting that some subset of CSF plasma cell Abs may be pathologically relevant agents in MS.

SUBJECTS/MATERIALS AND METHODS

MS CSF-derived monoclonal recombinant Abs

CSF was obtained from patients following informed consent (Table 1) and included individuals with either relapsing-remitting (MS03-1, n = 14; MS05-3, n = 14) or relapsing-progressive (MS04-2 # 30; n = 8) disease. CSF from patient MS03-1 was obtained after their first clinical event. rAbs were derived from expanded MS and inflammatory control CSF plasma blast clones using protocols previously described [21]. Reflecting the bias of MS CSF IgG, all rAbs were expressed as full-length bivalent human IgG1 Abs containing a C-terminal Flag epitope. Human IgG1 rAbs generated from patients with subacute sclerosing panencephalitis (SSPE rAb 2B4) and chronic meningitis (IC05-2 # 2 and IC05-2 # 76 rAbs) served as isotype controls.

Tissue preparation and immunohistochemistry

Adult C57bl/6 mice were perfused with 4% PFA in phosphate-buffered saline (PBS), and the brain and spinal cord were subsequently removed, postfixed overnight in 4% PFA, and cryoprotected overnight in 20% sucrose, all at 4° C. Tissue was embedded in optimal cutting temperature (OCT) freezing media, and 6 – 10 µm cryostat sections collected on Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA). Human optic nerve and cerebellum were dissected at autopsy, fixed in 4% PFA, cryoprotected in 20% sucrose, and sectioned at 10 µm. Tissue was stored at –80°C until immunostaining.

Prior to immunostaining, tissue sections were thawed for 10 minutes, re-hydrated in PBS for 10 minutes, and blocked in PBS containing 3% bovine serum albumin (BSA), 2% normal goat serum (NGS), and 0.3% Triton-X100 for 1 hour. Commercial antibodies used for co-localization included mouse monoclonal antibodies against MBP (1:1000, Covance), NeuN (1:500, Millipore), aquaporin-4 (AQP4)(1:100, Santa Cruz) and β III-tubulin, (1:1000, Sigma Aldrich) rabbit monoclonal antibodies against glial fibrillary acidic protein (GFAP) (1:200, Sigma), and synaptophysin (SYP) (EMD Millipore, 1:250), a rabbit polyclonal antibody against activated-complement components 5b-9 (C5b-9) (1:100, CompTech) and anti-chicken serum against neurofilament heavy (1:5000, Neuromics) and light (1:1000, Neuromics) chains. All patient-derived rAbs were used at a final concentration of 10 – 20 µg/mL. Primary antibodies were applied to mouse tissues for 16 hours at 4° C in PBS containing 3% BSA and 2% NGS. Tissue sections were then washed 3 times for 3 minutes with PBS, and species-specific fluorescent secondary antibodies (1:1000) against either human, mouse, rabbit or chicken IgG (Molecular probes, Life Science Technologies) were applied for 1 hour at room temperature in PBS containing 3% BSA and 2% NGS. Tissue was then washed 3 times for 3 minutes in PBS and mounted using Vectashield (Vector Laboratories, Burlingame CA) containing DAPI.

SuperBioChip Tissue arrays were utilized to characterize rAb reactivity toward peripheral targets. Arrays were deparaffinized and rehydrated through EtOH steps and into PBS. Antigen retrieval was utilized, slides were blocked in 10% NGS and 0.3% Triton-X100 for 1 hour. Primary antibodies were applied for 16 hours at 4° C in 10% NGS and 0.3% Triton-X100. Secondary antibodies were applied in 5% NGS, and slides were then washed in PBS containing 0.3% Triton-X100, followed by PBS and imaged as described.

For human tissue, binding of MS and control rAbs was monitored via their Flag epitope following our established protocol [21]. After primary incubation with rAbs as described above, tissues were washed 3 times for 5 minutes with PBS and fixed for 5 minutes in 4% PFA. Sections were again washed two times for 3 minutes with PBS and incubated an additional 10 minutes in blocking buffer containing 0.1% Triton-X100. Sections were next incubated for 1 hr with an anti-Flag mouse monoclonal Ab (1:1000 – 1:2000, Stratagene) in blocking buffer, washed again with PBS, and incubated for one hour in blocking buffer containing a 1:1000 dilution of goat anti-mouse IgG conjugated to Alexaflour 488 (Life Technologies). Sections were washed and mounted for microscopy as described above.

Neural cells and tumor cell lines

The U-87MG glioblastoma cell line was cultured on poly-ornithine-coated glass coverslips for 24 – 48 hrs, fixed with either ice-cold 4% PFA or acetone and stored at -20°C until staining. Dr. V Yong, University of Calgary, Canada, kindly provided primary human astrocytes. Astrocytes were cultured on poly-ornithine-coated glass coverslips for 24 – 48 hours in DMEM + glutamax containing 10% FCS, 1mM sodium pyruvate, and $1\times$ non-essential amino acids.

Human cortical neurons derived from induced pluripotent stem cells (iPSC; Cellular Dynamics International; Madison, WI) were cultured per manufacturer's instructions on poly-D-lysine/laminin coated glass coverslips (BD Biosciences; San Jose, CA). At 14 days post-plating, neurons were fixed in 4% PFA and prepared for immunofluorescence staining. For immunostaining, cells were re-hydrated in PBS followed by blocking for 1 hour in PBS containing 3% BSA and 2% NGS. The inclusion of Triton-X100 in blocking buffer produced no discernable effect on rAb binding and was not included in most experiments. Primary antibodies (5 – 20 $\mu\text{g/ml}$) were applied for 16 hours at 4°C in blocking buffer, washed four times in PBS, incubated with anti-human fluorescent secondary antibodies (1:1000) in blocking buffer for one hour, washed multiple times in PBS, and coverslips mounted using Vectashield plus DAPI.

Spinal Cord Slice Cultures

Swiss Webster outbred mice were obtained from Harlan Laboratories (Indianapolis, IN). All procedures were IACUC-approved and followed guidelines for the ethical treatment of animals. Spinal cords were removed from postnatal day 5–7 mice and 300 μm transverse spinal cord sections were cut using a McIlwain Tissue Chopper (Campden Instruments, UK). Thoracic and cervical slices chosen for culture were first evaluated for mechanical damage due to slicing. Undamaged slices were then evenly distributed into wells so that each treatment condition was applied to slices that were similar in size, quality, and myelin volume. Slices were placed on 0.4 μm 30 mm-diameter cell culture inserts in six well plates (5–6 slices per well) and allowed to recover in media containing 50% minimum essential medium, 25% Hank's Balanced Salt Solution, 25% horse serum, 1% Pen-Strep, .65% glucose, and 25 mM HEPES, pH 7.2 until postnatal day 17 as previously described [32]. On day 17, rAbs derived from MS patients and neuroinflammatory controls were applied to the media for 72 hours at concentrations of 50 $\mu\text{g/mL}$ in the presence and absence of 10% human complement (hComp) (pooled normal human complement serum; Innovative Research, Novi, MI). At 72 hrs, sections were fixed with ice-cold 4% PFA prior to immunostaining. Spinal cord slice data were derived from multiple experiments utilizing at least 3 separate litters. The number of slices evaluated for each condition is provided in the figure legend.

Scoring of Spinal Cord Slices for demyelination

MBP-immunostained spinal cord slices were evaluated by two, independent masked observers for lesion severity using the following scale: 0 = no MBP loss compared to culture media controls. 1 = 1%–20% MBP loss, 2 = ~ 21%–40% MBP loss, 3 = ~ 41%–60% MBP

loss, 4 = ~ 61%–80% MBP loss, 5 = about 81%–100% MBP loss. To assess significance, nonparametric ANOVA (Kruskal-Wallis) was performed followed by Dunn's posttest.

RESULTS

MS rAbs bind to neuronal and glial targets in CNS tissue

We first assayed individual Abs for immunoreactivity to brain-derived human tumor cell lines including U-87MG glioblastoma cells. In a comprehensive analysis of rAb binding to U-87MG cells, more than 50% of MS CSF-derived rAbs displayed some level of binding (summarized in Table 2) with many rAbs displaying a strong punctate or vesicular staining pattern (e.g., rAbs MS04-2 # 30, MS04-2 # 92, MS05-3 # 38, MS05-3 # 192, MS03-1 # 13, MS03-1 # 15 and MS03-1 # 57 in Figure 1a). Staining intensity and pattern changed from cell to cell based on the rAb. The localization of antigen varied from the perinuclear regions to the cell surface. Vesicular staining was sometimes observed outside the cell on the surrounding substratum. TIRF microscopy was used to visualize rAb binding at the cell surface (Figure 1b). In a sampling of three rAbs from different MS patients, surface staining was limited to discrete domains or puncta.

To determine whether autoreactive MS rAbs can also detect antigens in brain and spinal cord, rAbs demonstrating strong binding to the U-87MG glioblastoma cell line were used to stain freshly isolated and PFA-fixed mouse cerebral cortex, cerebellum, and spinal cord (Figs 2–4). MS04-2 # 30 exclusively co-localized with myelin markers in CNS tissue. We observed binding to MBP-positive (MBP+) myelinated fibers in deep white matter tracts and myelinated Purkinje cell axons in the mouse cerebellum (Fig 2a and Fig 2b), cerebrum (Fig 2c), and hindbrain (Fig 2d). MS04-2 # 30 binding was limited to neuronal β -III tubulin-positive axons in the myelinated white matter tract and granular layer of the cerebellum, indicating that MS04-2 # 30 antigen is expressed only around myelin and/or myelinated axons (Fig 2e). In addition, MS04-2 #30 stained MBP+ extensions emanating from spinal cord explant slice cultures (Fig 2f).

The majority of MS rAbs, as exemplified by MS05-3 # 38 rAb, displayed a second unique pattern of binding to CNS tissue. MS05-3 # 38 rAb showed strong immunoreactivity in the cerebellum. Binding was observed in the granular layer in close proximity to MBP+ myelinated Purkinje cell axons, in Purkinje cell bodies, and throughout the molecular layer (Fig 3a). Immunoreactivity co-localized extensively with an anti-synaptophysin mAb in the molecular but not granular layer of the cerebellum suggesting that rAb MS05-3 # 38 may be reactive against an antigen that is expressed strongly but not exclusively near synapses (Fig 3b–c). Additional MS rAbs (MS05-3 # 192, MS03-1 # 57 and MS03-1 # 15) displayed a similar distribution of immunostaining in the mouse cerebellum that also included some overlap with the astrocyte-specific protein AQP4 (Fig 3d), particularly evident in the larger astrocytic Bergman glia near the Purkinje layer. This panel of rAbs also stained neuronal cell bodies and neuropil in the mouse cortex and hippocampus (not shown). MS05-3 # 38 rAb immunostaining was visualized throughout the gray matter of the spinal cord, again co-localizing with synaptophysin (Fig 3e). Neuronal binding of spinal cord sections was confirmed by positive co-staining with synaptophysin (see Fig 3f). Confocal images of the

spinal cord further revealed binding to both GFAP+ astrocytes (see Fig 3g, middle panel) as well as GFAP- cells that appeared by morphology to be neuronal (see Fig. 3g, right panel).

MS03-1 # 13 rAb displayed a putative third pattern of CNS immunoreactivity. Although cerebellar staining patterns were similar to MS05-3 # 38, with broad, strong staining of neurons, astrocytes and neuropil (Fig 4a–c), MS03-1 #13 showed differential staining of neurons and human optic nerve (see below). Overlap with AQP4+ astrocytes was present throughout the cerebellum (not shown). MS03-1 # 13 staining was further distinguished by distinct nuclear staining of NeuN+ cerebellar granule cell neurons and NeuN+ neurons in the hippocampus, dentate gyrus, and cerebral cortex (Fig. 4, a–e). In the hippocampus, rAb MS03-1 # 13 bound to a substantial number of neuronal cells of the granular layer (Fig 4d), and to pyramidal neuron cell nuclei in layers CA1, CA2, and CA3 (not shown). MS03-1 # 13 also bound to a subpopulation of neurons throughout the cerebral cortex, notably in layers II/III (Fig 4e). Additionally, MS03-1 # 13 antigen was expressed on NeuN–ependymal cells surrounding the aqueduct of Sylvius (Fig. 4f) and on NeuN+ cells (not shown) throughout the spinal cord (Fig 4g). MS03-1 #13 rAb also stained aciniform MBP+ extensions emanating from the edges of spinal cord explant organ cultures (Fig 4h). The distribution of CNS tissue binding for MS rAbs is summarized in Table 3. Interestingly, while rAb binding was present in unique patterns in the CNS, MS04-2 and MS05-3 # 38 and rAbs also bound to epithelia cells in numerous tissues (supplemental Table 1; supplemental Figure 1).

We also examined the binding of MS CSF rAbs to human CNS tissue. Whereas isotype control failed to stain human cerebellum (Fig 5a), MS04-2 # 30 rAb bound myelinated tracts in both human cerebellum and optic nerve (Fig 5b and 5e). Co-staining with anti-neurofilament Abs showed MS04-2 # 30 rAb co-localized with cerebellar deep white matter tracts and myelinated Purkinje cell axons, but not with non-myelinated neuronal processes in the molecular layer. In human optic nerve, MS04-2 # 30 binding was restricted to MBP+ -positive (MBP+) myelinated fibers (Fig 5e). Similar to its staining of murine CNS tissue, MS05-3 # 38 recognized antigens expressed in cerebellar neurons and astrocytes (Fig. 5c). MS05-3 # 38 did not stain human optic nerve (not shown). Cerebellar staining of Purkinje cell neurons and adjacent Bergmann glia was evident as was diffuse staining of the molecular layer. Overlap in staining with AQP4+ astrocytes was evident throughout the cerebellum. Similar binding to human cerebellar neurons and astrocytes was observed with multiple MS rAbs (not shown) and was the dominant staining pattern observed among MS rAbs as summarized in Table 3. Although MS03-1 # 13 staining of human cerebellum revealed the same broad staining pattern as MS05-3 # 38, immunopositive staining of human cerebellar granule cell neurons was cytoplasmic, not nuclear as observed in mouse cerebellum (Fig 5d). Additionally, MS03-1 # 13 bound diffusely in a punctate pattern to AQP4+ cells in the human optic nerve and was particularly concentrated along connective tissue septa that divide nerve fiber bundles (Fig 5f). The specificity of most MS rAbs to antigens expressed by astrocytes and neurons was further validated in staining of primary human astrocytes and neurons (Fig 6). Greater than 50% of MS rAbs assayed (16/25) bound to cultured astrocytes (Table 2). 7 out of 9 astrocyte-positive MS rAbs that were further evaluated for neuronal binding were also positive including all MS rAbs demonstrated to bind astrocyte/neuronal targets in mouse and human CNS tissue (Table 3). Consistent with

its binding to myelin-enriched antigen, MS04-2 # 30 bound poorly to cultured astrocytes and failed to bind primary human neurons.

MS rAbs promote demyelination in spinal cord explant cultures

To determine whether CNS-reactive MS rAbs contribute to CNS demyelination, we next examined the effect of MS rAbs with human hComp on spinal cord explants. Three rAbs that were derived from brain or CSF plasma cells of patients with other CNS inflammatory diseases (2B4, IC05-2 # 2 and IC05-2 # 76) were used as isotype controls. Spinal cord sections treated with either rAbs MS04-2 # 30 or MS05-3 # 38 and hComp showed almost complete loss of myelin as measured by MBP immunostaining (Fig 7a and 7b). By comparison, the isotype control rAbs did not induce any more myelin loss than observed in spinal cord sections maintained in culture medium alone. Although some tissue damage and myelin loss was observed in sections treated with rAb MS03-1 # 13 + hComp, it did not reach statistical significance.

MS rAbs cause damage via complement-dependent cytotoxicity

To determine whether or not demyelination was solely dependent on complement-dependent cytotoxicity (CDC), we compared the extent of tissue damage in sections treated with MS rAbs in the presence and absence of hComp (Fig 8a). MBP loss was significantly enhanced for MS04-2 # 30- and MS05-3 # 38-treated sections in the presence of hComp. The inclusion of hComp had no measurable effects on sections treated with isotype control rAb or hComp in the absence of Abs. To document the presence of terminal complement activation, spinal cord sections were next immunostained for complement proteins C5b-9 (Fig 8b), indicating formation of membrane attack complexes (MACs). C5b-9 deposition was not observed in culture media treated sections with or without hComp or sections treated with rAbs IC05-2 # 2 and MS03-1 # 13 in the presence of hComp, but was clearly prominent in spinal cord sections treated with rAbs MS04-2 # 30 and MS05-3 # 38 (Fig 8c).

MS rAbs causing CNS damage also result in astrocyte activation

Astrogliosis is a well-defined feature of MS tissue damage. To assess astrocyte reactivity in explant cultures, sections treated with MS and isotype control rAbs in the presence of hComp were immunostained with GFAP and evaluated by microscopy (Fig 9). Compared to baseline GFAP expression in untreated spinal cord sections (culture media), IC05-2 # 2-treated and MS03-1 # 13-treated spinal cord culture showed no evidence of enhanced GFAP expression. In contrast, explants incubated with rAbs MS04-2 # 30 and MS05-3 # 38 contained astrocytes with strongly enhanced GFAP staining. By confocal microscopy, astrocytes in untreated and IC05-2 # 2- and MS03-1 # 13-treated sections had long and thin GFAP+ astrocytic process relative to the shortened intense ramified processes observed in MS04-2 # 30 and MS05-3 # 38-treated spinal cords. Astrocyte activation in rAb MS05-3 # 38-treated sections was particularly striking where astrocytes with large cell bodies and thick processes were found throughout areas of partial demyelination.

DISCUSSION

We previously described the generation, production, and analysis of monoclonal rAbs from clonally expanded plasma blasts isolated from the CSF of multiple sclerosis patients [21]. Herein, we have employed new approaches to define the CNS target(s) and pathogenicity of MS CSF rAbs reproduced from the intrathecal antibody response in affected individuals. Using human glial tumor cell lines, cultured primary human astrocytes and neurons, and freshly-isolated and PFA-fixed mouse and human CNS tissue, we have identified MS rAbs with distinct CNS staining patterns that can mediate demyelination and CNS tissue injury.

The majority of MS rAbs (26/33) we evaluated in this study bound to human U-87MG glioblastoma cells in a characteristic punctate pattern. Although further staining of CNS tissue revealed both myelin and neuronal/astrocytic targets, a surprisingly large fraction of MS rAbs were specific for antigens expressed by astrocytes and neurons. This was observed in both mouse and human CNS tissue and confirmed by staining of cultured primary human astrocytes and neurons (Table 2). The large fraction of MS rAbs binding to cultured human astrocytes (16/25) indicates that the bulk of plasma blasts in this sampling of MS patients are likely targeting astrocytic/neuronal targets and not myelin antigens. Indeed, the number of MS rAbs revealing this pattern of CNS tissue staining (Table 3) further indicates that responses against astrocyte and neuronal targets dominate the intrathecal B cell response. The relevance of neuronal nuclear staining observed with rAB MS03-1 #13 (Table 3) remains to be determined and differences in nuclear staining on mouse and human CNS tissue may be due to tissue preparation. Fixation conditions likely play an important role as 4% PFA fixation preserved epitopes in mouse and human tissue, whereas previous immunostaining of formalin-fixed and paraffin embedded human tissue showed scant MS rAb immunoreactivity. [21]

The rAb MS04-2 # 30 binds to myelinated tracts in both brain and spinal cord in a pattern prototypical of myelin proteins as shown by co-localization with MBP (Figs 2 and 5) and MOG (data not shown). This myelin-reactive binding is reminiscent of immunostaining observed in an independent study of other MS CSF-derived rAbs [29], although in that study binding was more prominent in white matter tracts at the edge of active MS lesions. The antigens recognized by this set of myelin-reactive antibodies remain unknown, and attempts to correlate myelin specificity to cell lines expressing individual myelin-enriched proteins (MBP, PLP, MOG, CLDN11, CNP, PLPP, MAL1, MAL2) have been negative ([21] and unpublished data). Additional efforts to characterize the binding of MS CSF- and inflammatory control CSF-derived rAbs to myelin-enriched glycolipids have also proven confounding. Although both MS CSF IgG and a subset of MS rAbs bind to myelin-enriched glycolipids, particularly complexes of sulfatides and galactose cerebrosides, no significant binding to myelin-enriched glycolipids was observed for rAb MS04-2 # 30 [8].

MS lesion formation is characterized by early inflammation and demyelination followed by astrogliosis. The composition of inflammatory cell infiltrates in MS lesions is variable, but generally includes activated microglia, macrophages, oligoclonal T- and B-lymphocytes, and occasional plasma cells [24,28]. Abundant axonal transections observed within acute MS plaques indicate that the underlying pathology is not limited to myelin and that axonal

injury and neurodegeneration, particularly of the corticospinal tract, may be primary contributors to tissue injury and long-term disability [27,16]. In the majority of active MS lesions, tissue histopathology reveals deposition of immunoglobulins and terminally activated complement in association with phagocytic macrophage activity at the plaque margins [18,7], suggesting that antibodies may participate directly in CNS lesion formation. In this study, we chose to use ex vivo spinal cord sections to evaluate the pathogenicity of MS CSF-derived rAbs since this model system has been used previously to assess demyelination and CNS damage following exposure to anti-AQP4 antibodies [32]. We found that rAbs MS04-2#30 and MS05-3#38, but not inflammatory control CSF-derived rAbs, recapitulated several pathologic features characteristic of active MS lesions including demyelination as measured by loss of MBP immunoreactivity, activation of terminal complement complexes as visualized by C5b-9 immunostaining, and astrogliosis [7,18].

Although spinal cord sections offer a quick and measurable read-out for extensive Ab-mediated myelin loss in a disease-relevant tissue, there are limitations to what can be inferred regarding mechanisms of disease. An incomplete presence of myelin, as measured by MBP immunostaining, appears to be inherent to the protocol and is observed in some spinal cords simply maintained in culture media alone (see Figure 5), thereby creating a high threshold for the detection of meaningful myelin loss. Despite this caveat, the damage produced by MS04-2 # 30 and MS05-3 # 38 rAbs is extensive and far above that observed in control experiments. MS03-1 # 13 rAb may produce CNS damage that is below the threshold of detection in the spinal cord slice; however, the extensive complement deposition and astrocyte activation observed with rAbs MS04-2 # 30 and MS05-3 # 38 is not evident. For these experiments, rAb MS03-1 # 13 is expressed as a human IgG1 molecule. Interestingly, the native MS03-1 # 13 plasma blast clone from which this rAb was derived was actually a human IgG3 subtype. Because human IgG3 is more efficient than IgG1 in activating human complement, rAb MS03-1 # 13 might have caused more damage if expressed in its native form.

In the spinal cord slice assay, the preparation of the spinal cord slices precludes further analyses of neuronal loss and axonal transections as axonal damage and neuronal loss from antibody-mediated injury would be difficult to discern from damage produced during tissue preparation. Additionally, the anatomy of cultured postnatal spinal cords remains immature and does not mirror that found in adulthood and thereby limiting a more detailed study of primary and secondary mechanisms of damage. Lastly, the slice model cannot duplicate the infiltration of inflammatory cells observed in MS lesions, particularly actively demyelinating phagocytic macrophages. Reproducing the myriad features of MS pathology, including the loss of vulnerable neural cell populations, will likely require in vivo models of disease. Nonetheless, the spinal cord slice assay clearly establishes that antibodies produced by MS CSF plasma cells mediate complement-dependent CNS demyelination ex vivo.

In this study, two of three MS rAbs caused significant loss of myelin in spinal cord sections. Whether antibodies promoting CNS demyelination are rare in MS CSF or represent the majority of clonally expanded plasma blasts, akin to AQP4-specific plasma blasts in neuromyelitis optica, is unknown. MS CSF-derived rAbs examined for pathogenicity in this study were chosen because they readily stained both mouse and human CNS tissue. A focus

of future studies will determine if most Abs binding to neuronal and astrocytic targets (Group 1, Table 3) are equally pathogenic. The mechanism by which MS04-2 # 30 rAb causes damage most likely entails binding to a myelin-enriched target antigen. Whether binding occurs to an exposed myelin antigen or requires some prior insult, such as damage initiated by spinal cord sectioning, remains unknown. More curious is the pathway leading to myelin loss in rAb MS05-3 # 38-treated sections, since this Ab binds to astrocytes and neuronal populations, yet causes demyelination and complement terminal complex formation in a manner similar to a myelin-directed rAb. However, this finding is not without precedent. Antibodies directed against the neuronal protein Neurofascin 186 cause some axon loss in culture (~15%) followed by complete, but secondary, complement-dependent demyelination [11]. Likewise, pathogenic Abs directed against the water channel AQP4 that is prominent in astrocytic end feet initiate CNS demyelination. Both in vivo and ex vivo spinal cord slice cultures reveal complement-mediated killing of astrocytes followed by oligodendrocyte cell death and demyelination [3,6,25,32]. Therefore, like the pathology driving demyelination in NMO, immunologic injury to the cellular partners of oligodendrocytes might play an important role in MS pathophysiology.

Our observation that some MS rAbs can bind to neuronal targets is intriguing. Neuronal pathology in MS has been investigated extensively since Trapp and colleagues demonstrated the prevalence of axonal transection in MS lesions [27]. Axopathic and/or demyelinating IgG preparations derived from MS patients have been described, and MS-associated autoantibodies have been observed to cause demyelination in a cell culture myelination model [11]. IgG autoantibodies against the inward-rectifying potassium channel KIR4.1 have been detected in the serum of 47% of the 397 MS patients tested [26]. When KIR4.1 antibody-containing sera were injected into mouse cerebellum, changes in astrocyte morphology and complement activation were evident 24 hours post-injection. Damage to myelin, however, was not reported.

In conclusion, our data provide evidence that rAbs derived from MS CSF B cells are directed against targets in human CNS tissue that are predominantly expressed by astrocytes and neurons. Furthermore, these rAbs can mediate damage in a spinal cord explant model with features of MS histopathology: loss of myelin, astrocyte activation, and deposition of complement products. If intrathecal IgG production contributes significantly to MS pathogenesis, then these data suggest that the CNS targets of a patient's intrathecal IgG profile may be important in determining the breadth and severity of tissue injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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a. Fluorescence Microscopy

b. TIRF Microscopy

Figure 1. Glial tumor cell stainings

Human U-87MG glioblastoma cells were immunostained with isotype control antibody and the indicated MS CSF-derived rAbs. Overall 26/33 MS rAbs assayed for binding were immunopositive relative to isotype controls. A summary of MS CSF-derived rAb binding to U-87MG cells is listed in Table 2. Bar in IC image equals 25 μ m and represents magnification of all images..

Figure 2. MS04-2 # 30 binds white matter antigen throughout the CNS

(a–b) Panels show sagittal sections (a, scale bar is 200 μm ; b, scale bar is 20 μm) of mouse cerebellum immunostained with MS04-2 # 30 and MBP. Merged images show that MS04-2 # 30 antigen co-localizes strongly with MBP in the cerebellar white matter and stains in a punctate pattern in the granular layer. (c) Mouse cerebellar lobule immunostained with MS04-2 # 30 and a mAb against the neuronal marker β -III tubulin (bar equals 100 μm). There is strong colocalization of MS04-2 # 30 antigen and β -III tubulin in the white matter and granular layer, but not in the molecular layer. (d) Coronal section (bar equals 200 μm) of

the mouse cerebrum, and (e) sagittal section (bar equals 50 μ m) of the hindbrain demonstrate co-localization of MS04-2 # 30 antigen with MBP. (f) Outgrowths from the edge of spinal cord explant cultures (bar equals 30 μ m) reveal the juxtaposition of MS04-2 # 30 and MBP staining.

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Figure 3. MS CSF-derived rAbs bind CNS neurons and astrocytes

(a) Mouse cerebellum immunostained with MS05-3 # 38 rAb and anti-MBP mAb as indicated. Merged image is to the right. MS05-3 # 38 antigen expression was low in the deep white matter, but was readily observed in close proximity to myelinated axons in the granular layer, in Purkinje cell bodies, and in the unmyelinated molecular layer. Scale bar equals 400 μ m. (b and c) Sagittal cerebellum sections were immunostained with MS05-3 # 38 rAb and an anti-synaptophysin mAb (SYP). A strong overlap between MS05-3 # 38 and SYP staining was noted in the molecular layer and in Purkinje cell bodies, but not in the

granule cell layer of the cerebellum. Scale bar equals 25 μm (d) Sagittal cerebellum sections stained with isotype control or MS CSF rAb (green), in combination with an astrocyte-specific anti-AQP4 mAb (AQP4, red). MS CSF rAbs MS05-3 #92, MS03-1 #57, and MS03-1 #15 display a similar neuronal staining pattern to that observed with MS05-3 #38 rAb. Some staining overlaps with AQP4+ astrocytes (arrows). Scale bar equals 40 μm . (e) Spinal cord sections stained with MS05-3 #38 rAb, and SYP demonstrate MS05-3 #38 antigen predominately in the gray matter. Scale bar equals 250 μm (f) Magnification of the overlap between MS05-3 #38, and SYP expression in mouse spinal cord. Scale bar equals 50 μm (g) *Left*, Spinal cord sections stained with MS05-3 rAb (green) and glial fibrillary acidic protein (red). Scale bar equals 200 μm . Confocal images reveal MS05-3 #38 rAb staining (green) of both GFAP+ (red) astrocytes (*middle*) and GFAP- cells (*right*) that appear by morphology to be neuronal. Scale bar equals 10 μm .

Figure 4. MS03-1 # 13 binds neuronal targets throughout the
 (a) Sagittal section of mouse cerebellum immunostained with MS03-1 # 13 rAb and an anti-NeuN mAb showing nuclear and/or perinuclear co-localization between NeuN and MS03-1 # 13 antigen in the granular layer and scattered staining of NeuN– nuclei in the molecular layer. Scale bar = 100 μ m (b) Sagittal cerebellar section immunostained with MS03-1 # 13 rAb and anti-MBP Ab showing little co-localization between MS03-1 # 13 antigen and MBP + myelinated axons in the white matter or granule layer of the cerebellum. Scale bar = 200 μ m (c–d) Mouse hippocampal sections stained with DAPI, MS03-1 # 13 rAb and anti-NeuN

Ab. MS03-1 # 13 antigen co-localizes strongly, and in a punctate pattern, with NeuN+ neurons in the granular layer (cropped and enlarged in row d). Scale bar in C = 40 μ m. Scale bar in D = 15 μ m (e) Mouse cerebral cortical layers II/III demonstrate a similar colocalization of MS03-1 # 13 antigen and NeuN. Scale bar = 60 μ m (f) Mouse cerebrum immunostained with MS03-1 #13 rAb and anti-NeuN show expression of MS03-1 # 13 antigen on NeuN- cells surrounding the Sylvian fissure. Scale bar = 200 μ m (g) In adult mouse spinal cord, MS03-1 # 13 antigen was expressed in cell bodies. Scale bar = 400 μ m. (h)) Outgrowths from spinal cord explants immunostained with MS03-1#13, and MBP. MS03-1 # 13 antigen shows aciniform pattern on extending processes. Scale bar = 30 μ m.

Figure 5. MS rAb binding to human cerebellum and optic nerve

(a) Images of isotype control rAb and anti-AQP4 specific rabbit IgG binding to human cerebellum. The limited green fluorescence in rAb channel represents modest tissue autofluorescence and not IC05-2 # 2 binding. Size bar equals 100 μ m. (b) Images show binding of MS04-2 # 30 to myelin-enriched antigen in cerebellar deep white matter and granule cell layer. Co-localization with neurofilament is only noted on myelinated processes, but not in cerebellar molecular layer. Size bar equals 100 μ m. (c) MS05-3 # 38 binding to astrocytes, Purkinje neurons and the molecular layer of cerebellum. Co-localization with

AQP4+ astrocytes is evident throughout the cerebellum. Size bar equals 100 μ m. (d) Images reveal binding of MS03-1 # 13 rAb to cerebellar neurons and astrocytes. Binding to neuronal nuclei in human cerebellum was not observed. Size bar equals 100 μ m. (e) Confocal images showing binding of MS04-2 # 30 to MBP+ myelin in human optic nerve. Asterisk shows the absence of binding to cells in the optic nerve septa. Size bar equals 25 μ m. (f) Confocal images show overlap between MS03-1 # 13 rAb and AQP4 staining throughout human optic nerve. Asterisk shows strong co-localization between MS03-1 # 13 and AQP4 in the optic nerve septa. Scale bar equals 25 μ m.

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a. Human neurons

b. Human astrocytes

Figure 6. MS rAb binding to cultured human neurons and astrocytes

(a) Images show binding of numerous MS rAbs, as indicated, to primary human neurons. Staining with β -III tubulin was used as a positive control. Scale bar shown in isotype control (IC) immunostaining equals 50 μ m and represents magnification of all staining images. (b) Images show binding of numerous MS rAbs, as indicated, to primary human astrocytes. A majority of MS rAbs showed binding to human astrocytes as summarized in Table 2. GFAP staining was used as a positive control. Bar shown in isotype control (IC) equals 50 μ m.

Figure 7. MS rAbs mediate spinal cord demyelination

(a) 300 μ m transverse spinal cord slices (n=42) were incubated in culture media, and then 50 μ g/ml of the following rAbs were added in the presence of 10% human complement (hComp): isotype control antibody IC05-2 # 2, (n = 10), isotype control antibody 2B4, (n = 13), isotype control antibody IC05-2 # 76 (n = 13), MS rAb MS03-1 # 13 (n = 7), MS rAb MS04-2 # 30 (n = 9), or MS rAb MS05-3 # 38 (n = 21). Each row from top to bottom shows DAPI counterstain, MBP immunostaining, and the merged DAPI (blue) and MBP (red) image. MBP reactivity is significantly reduced in MS04-2 # 30- and MS05-3 # 38-treated sections. (b) MS04-2 # 30 or MS05-3 # 38 rAb and hComp resulted in a significant loss of MBP (**p<0.01; *** p < 0.001; nonparametric ANOVA with Dunn's posttest).

Figure 8. Complement activation in MS Ab-mediated spinal cord demyelination

(a) Loss of MBP immunostaining was compared in spinal cord sections exposed to either isotype control or MS rAbs in the presence and absence of human complement (hComp). The addition of hComp caused no significant loss of MBP in spinal cord sections treated with isotype control IC05-2 #2 rAb ($n = 7$, no hComp; $n = 14$ plus hComp). Although the addition of complement appeared to decrease MBP staining in sections treated with MS03-1#13 rAb ($n = 8$, no hComp; $n = 10$, plus hComp), differences did not reach statistical significance. The addition of complement to spinal cord sections treated with MS04-2 #30 ($n = 12$, no hComp; $n = 14$, plus hComp) or MS05-3 #38 ($n = 8$, no hComp; $n = 12$, plus hComp) rAbs resulted in significant loss of MBP. (* $p < 0.05$; *** $p < 0.001$; nonparametric ANOVA with Dunn's posttest). (b) Spinal cord slices grown in culture media or treated with 50 µg/ml of IC05-2 #2 rAb, MS03-1 #13 rAb, MS04-2 #30 rAb, or MS05-3 #38 rAb for 72 hr without hComp showed no evidence of membrane attack complex (MAC) deposition

using anti-C5b-9 immunostaining. (c) Spinal cord slices grown in culture media with hComp only or incubated with 50 µg/ml of IC05-2 # 2 rAb, MS03-1 # 13 rAb, MS04-2 # 30 rAb, or MS05-3 # 38rAb in the presence of 10% hComp show MAC deposition only after exposure to rAbs MS04-2 # 30 or MS05-3 # 38 (anti-C5b-9; green). Exposure to rAb MS03-1 # 13 with hComp shows no significant MAC deposition. Some very modest C5b-9 deposition was noted at the edge of spinal cord exposed to control 2B4 rAb.

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Figure 9. MS rAbs + hComp activate astrocytes

Spinal cord slices were incubated in culture medium + hComp or with human rAbs (50 ug/ml) and 10% hComp as described, fixed in 4% PFA and immunostained with an anti-GFAP mAb. Top image for each condition represents a low magnification image, scale bar = 100 μ m. Lower image for each condition represents confocal image for each condition, scale bar = 30 μ m. In the presence of MS04-2 # 30 or MS05-3 # 38 and hComp, astrocyte cell bodies were larger, and processes more highly arborized, than in culture media alone or following treatment with IC05-2 # 2 and hComp. Exposure times for GFAP+ green

fluorescence in upper MS04-2 # 30- and MS05-3 # 38-treated slices were reduced compared to control and MS03-1 #13 images to prevent overexposure and to satisfactorily show astrocytic processes.

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Clinical, MRI and CSF Features of MS Patients used for CSF monoclonal recombinant antibody production^a

Table 1

Patient	Age	Sex	Diagnosis	White Matter Lesions	CSF Cells/ μ l	IgG Index	OCBs	Disease Duration at Lumbar Puncture	Time of Lumbar Puncture from Last Exacerbation
MS03-1 ^b	25	F	RRMS	5	0	1.4	6	6 months	2 months
MS04-2	26	F	RPMS	36	0	1.7	5	6 months	1 month
MS05-3	30	F	RRMS	4	18	1.7	21	14 months	2 months

^aPatients were not receiving immunomodulatory therapy at the time of lumbar puncture. RRMS, relapsing-remitting multiple sclerosis. RPMS, relapsing-progressive multiple sclerosis

^bCSF was collected and analyzed following a first clinical event. Patient subsequently developed relapsing-remitting MS within a 6-month period. MS patient's CSF (MS03-1) repertoire was originally reported in Ritchie et al.⁸

Table 2

Summary of MS CSF-derived rAbs assayed for binding to cultured CNS cells and lines

MS CSF Donor ^a	U-87MG Glioblastoma Tumor Cell Line		Human Astrocytes		Human Neurons ^b	
	# rAbs Assayed	# Positive rAbs	# rAbs Assayed	# Positive rAbs	# rAbs Assayed	# Positive rAbs
MS03-1	11	8	9	6	3	3
MS04-2	8	7	6	4	2	1
MS05-3	14	11	10	6	2	2

^a Antibodies used in this study were derived from patients with relapsing-remitting (MS03-1 and MS05-3) or relapsing progressive (MS04-2) multiple sclerosis including CSF obtained after their first clinical attack (MS03-1). Binding assays were performed on U-87MG human glioblastoma tumor cell line, cultured primary human astrocytes cells grown on glass coverslips or differentiated primary human neurons as described in Methods. Positive immunoreactivity was based on comparison to isotype controls imaged under identical conditions as shown in Figure 1 and 6 for some MS CSF-derived rAbs.

^b Because of limited samples, only MS rAbs shown to immunostain CNS tissue or human astrocytes were assayed for binding. Among MS rAbs demonstrated to bind mouse or human CNS tissue, only MS04-2 # 30 failed to stain neurons consistent with its myelin-specific staining pattern.

Table 3Patterns of MS CSF-derived rAb binding to Human CNS tissue^a

CNS Binding Patterns	MS CSF rAb	Description
1. Astrocytes and Neurons	MS03-1 # 15 MS03-1 # 17 MS03-1 # 57 MS05-3 # 38 MS05-3 # 39 MS05-3 # 192 MS04-2 # 92	Dominant binding pattern with staining of murine and/or human astrocytes, neurons and their processes in CNS tissue sections.
2. Myelin-enriched antigen	MS04-2 # 30	Binding co-localizes with myelin-specific markers only in murine and human CNS tissue.
3. Astrocytes and Neurons	MS03-1 # 13	Similar CNS binding as Group 1, but distinguished by clear binding to cerebellar granule cell neurons and septa in human optic nerve. The binding to neuronal nuclei observed in mouse tissue was not observed in immunostaining of human cerebellum.

^a All antibodies summarized in this section were shown to bind antigens in human CNS tissue. Some but not all rAbs were also assayed for binding to mouse CNS tissue as described in the manuscript. This table represents immunostainings on human cerebellum for 9/10 MS rAbs. Abs were pre-selected for evaluation based on previous staining of murine tissue or from those Abs shown to bind cultured primary human astrocytes.