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Age-Dependent B Cell Autoimmunity to a Myelin Surface Antigen in Pediatric Multiple Sclerosis


Multiple sclerosis (MS), a chronic inflammatory demyelinating disease of the CNS, is the most common cause of chronic neurological disability in young adults (1). There has been increasing recognition of pediatric MS over the past two decades, in part due to the advent of magnetic resonance imaging (MRI) techniques, which enable sensitive detection of CNS white matter abnormalities (2). It is now estimated that 2–5% of cases of MS begin before an age of 16 (3–5), but the true incidence of pediatric MS remains unknown. The average age of clinical onset in most pediatric MS cohorts is between 8 and 14 years, but the disease occurs even in children 2–5 years of age. In the majority of pediatric MS patients (>90%), the disease course is relapsing-remitting. Pediatric disease onset is associated with a younger age at disease progression milestones, such as the requirement for mobility aids, than adult-onset MS (4–7). The rate of disease relapses is significantly higher in pediatric-onset MS than adult-onset disease, even when the initiation of disease-modifying therapies following a clinically isolated event is taken into account (8). A short interval (less than 1 year) between the first two demyelinating episodes, incomplete recovery after the first attack as well as a secondary progressive disease course are unfavorable prognostic factors (9, 10). Cognitive impairment is common in pediatric-onset MS, and can seriously affect academic performance (11–13).

Unlike adult-onset MS, which is more common in females and populations with European ancestry, pediatric MS occurs in many ethnic groups and does not have a strong gender bias before puberty (14, 15). An analysis of MS patients cared for in Toronto showed that parents were of non-European descent for a substantially larger percentage of pediatric MS patients (41.9% of mothers and 48.8% of fathers) than for adult-onset MS patients (8.1% of mothers and 7.1% of fathers), and represented ethnic groups from Asia, the Caribbean, and the Middle East (15). Similar to these findings, a recent study in the northeastern United States revealed that only 86.2% of patients with pediatric-onset MS identified themselves as Caucasian, compared with 93.1% of patients with adult-onset MS (p = 0.014) (3). Specifically, the proportions of African-American and Latin-American patients were higher in the pediatric-onset group.

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2 Address correspondence and reprint requests to Dr. Kai W. Wucherpfennig, Dana-Farber Cancer Institute, 44 Binney Street, Room D1410, Boston, MA 02115. E-mail address: Kai.Wucherpfennig@dfci.harvard.edu.

3 Abbreviations used in this paper: MS, multiple sclerosis; ADEM, acute disseminated encephalomyelitis; CIS, clinically isolated syndrome; CSF, cerebrospinal fluid; MAG, myelin-associated glycoprotein; MFI, mean fluorescence intensity; MOG, myelin oligodendrocyte glycoprotein; NMO, neuromyelitis optica; OMG, oligodendrocyte-myelin glycoprotein; MRI, magnetic resonance imaging; HA, hemagglutinin.
Early during the disease course, there can be substantial difficulties distinguishing between pediatric MS and acute disseminated encephalomyelitis (ADEM) (16). ADEM is characterized by an acute onset of widespread CNS inflammation and demyelination and is more common in children than adults (16–18). For a diagnosis of ADEM, new criteria established by the International Pediatric MS Study Group require a polysymptomatic clinical presentation that includes encephalopathy, which is defined as either a behavioral change (such as confusion, excessive irritability) or alteration in consciousness (lethargy, coma) (19). ADEM typically follows a monophasic course with partial or complete recovery. However, recurrent ADEM (defined as a new event of ADEM with a recurrence of the initial symptoms 3 mo or more after the initial event) and multiphasic ADEM (two or more distinct ADEM episodes, each meeting clinical criteria for ADEM but involving new areas of the CNS, separated by at least 3 mo) occur rarely (20). Because the prognosis and treatment regimens for MS and ADEM differ substantially, it is essential to understand the biological similarities and differences between these conditions.

The largest reported study of pediatric demyelinating diseases illustrates this issue (21). In this study, 296 patients were followed after an initial demyelinating episode: 168 patients (57%) experienced two or more episodes of demyelination and were given a diagnosis of MS. Although MS was more commonly preceded by a clinically isolated syndrome (CIS), 20% of the patients with a final diagnosis of MS were considered to have ADEM at clinical onset. Given these diagnostic difficulties, biomarkers that reflect differences in disease pathogenesis would be valuable. For example, Abs to aquaporin-4, a water channel enriched on astrocytic foot processes at the blood-brain barrier, are commonly found in neuromyelitis optica (NMO), an inflammatory demyelinating disease that affects the optic nerves and the spinal cord (22). Higher Abs are associated with increased disease activity (23), and in some patients, and CSF analysis of 136 patients with a disease onset before age 16 identified oligoclonal IgG in 92% of cases (26), but the specificity of these Abs remains unknown.

Pathogenic autoantibodies need to be able to bind to structures on the surface of the myelin or oligodendrocytes, but the abundant myelin Ags myelin basic protein and proteolipid protein are inaccessible to Abs in the intact myelin sheath. Myelin oligodendrocyte glycoprotein (MOG), a CNS-specific myelin and oligodendrocyte surface protein, is one of the relevant candidate Ags, and a mAb (8-18C5) against MOG induces severe demyelination in mice or rats with mild experimental autoimmune encephalomyelitis (27, 28). Although Abs able to bind to the native conformation of the protein cause demyelination, Abs to MOG peptides that do not bind the folded protein are not pathogenic (29, 30). It is therefore critical to differentiate between Abs to folded MOG and denatured or linear epitopes, a task for which traditional assays such as Western blot and ELISA analyses are poorly suited.

We recently reported a radioimmunoassay with a tetrameric form of MOG for the detection of MOG autoantibodies in patients with inflammatory demyelinating CNS diseases and found Abs to MOG in a subset of patients with ADEM (13/69 patients, 18.8%) (31). Interestingly, one of 19 pediatric MS sera gave a strong signal in this radioimmunoassay, whereas only one of 109 adult-onset MS sera was positive. This pediatric MS serum sample also labeled a MOG-GFP transfected, indicating that autoantibodies in this patient indeed bound native MOG. We therefore decided to examine a larger cohort of pediatric and adult-onset MS patients to define the relationship between the presence of such autoantibodies and age at disease onset.

Materials and Methods

Clinical samples

Pediatric and adult samples were collected by international neurological centers that care for adults or children with MS and other demyelinating diseases. Pediatric MS was defined using the McDonald Criteria (32, 33). None of the cases defined as pediatric MS met the criteria for multiphasic or recurrent ADEM (19). The majority of pediatric-onset MS and pediatric control serum samples were obtained from centers participating in the Wadsworth International Pediatric MS Consortium, which did not make MRI scans available for centralized review. Adult-onset MS was defined using the Poser or McDonald criteria (32–34). Most adult-onset MS (including relapsing-remitting, secondary progressive, and primary progressive MS), CIS, and control serum and CSF samples were collected at the Partners Multiple Sclerosis Center at Brigham and Women’s Hospital (Boston, MA). Serum samples from patients with NMO were from the Hôpital Civil (Strasbourg, France), and viral encephalitis samples were provided by the New York State Department of Health. Each site collected samples using a protocol approved by their Institutional Review Board, and informed consent was obtained from all subjects. Samples were stored at −80°C in aliquots.

Selection of MOG-GFP clones

The cloning of human MOG into the pEFGP-N1 vector (Clontech) and transfection of Jurkat cells was previously described (31). Transfected cells were grown in DMEM (Invitrogen) supplemented with 10% FBS (Atlanta Biologicals), 100 IU/ml penicillin, 100 μg/ml streptomycin, 35 mM HEPES, and 2 mM l-glutamine (Meditech) with 1 mg/ml G418 (Invitrogen) to maintain selection. MOG-GFP and GFP clones were generated by sorting single GFPpositive cells on a FACSＡria (BD Biosciences). After expansion, MOG clones were stained with an Ab to MOG (clone 8-18C5), and MOG-GFP and GFP clones with a matching level of GFP brightness were selected.

Flow cytometry

Cells were harvested, washed in PBS containing 1% BSA (Fisher) (PBS/ BSA), and resuspended at a density of 1 × 10⁶/ml. A total of 50,000 cells (50 μl) were incubated with serum at a 1/50 dilution (or other, as indicated) in V-bottom plates (Corning) for 1 h at 4°C. For CSF stains, 25,000 cells were used. Abs were used at a density of 2 × 10⁵/ml and incubated with 50 μl of CSF. Cells were washed twice with 200 μl of PBS/BSA, and incubated with biotinylated secondary Abs to human IgG (pan-IgG clone HP-6017 (Sigma-Aldrich), or subclass-specific IgG1 clone HP6007, IgG2 clone HP6002, IgG3 clone HP6067, and IgG4 clone HP6023 (Calbiochem)) at a 1/1000 dilution for 30 min at 4°C. Cells were washed twice and incubated with streptavidin-PE (Invitrogen) at a 1/10000 dilution for 20 min, washed once with PBS alone and fixed with 1% formaldehyde in PBS at 4°C before analysis. A total of 10,000 events per well were recorded on an LSR II instrument equipped with a high-throughput sampler (BD Biosciences). Data analysis was performed in FlowJo (Tree Star) and Excel (Microsoft). Nonparametric statistical tests were performed in SISA Tables (Quantitative Skills).

Affinity purification of Abs to MOG for FACS analysis

The Ig domain of MOG and the membrane proximal Ig domain of CD80 were linked to a BirA biotinylation site and cloned into the pET22b vector (Promega). Proteins were expressed in Escherichia coli, refolded from inclusion bodies, purified by HPLC, and biotinylated using the BirA enzyme using published protocols (35, 36). A total of 100 μg of biotinylated MOG or CD80 was incubated with 50 μl of settled streptavidin-agarose beads (Sigma-Aldrich) in 200 μl of PBS/BSA overnight at 4°C on an orbital rotator, and washed to remove unbound Ag. The 10 μl of settled beads were resuspended with 5 μl of CSF and 200 μl of PBS/BSA at 4°C. The supernatant was saved, and the beads were washed four times with 1 ml of PBS/BSA, followed by elution in 200 μl of 0.1 M glycine (pH 2.5). Eluted proteins were separated from the beads by spin filtration (Spin-X 0.2
immunoperoxidase staining was performed with a Vectastain avidin-biotin peroxidase kit (Vector Laboratories) using diaminobenzidine and nickel (II) chloride to give black staining (37). Staining patterns observed with patient IgG were compared with adjacent sections labeled with the myelin-specific anti-MOG mAb 8-18C5 and the oligodendrocyte-specific mAb 14E (38). Primary Abs were omitted for immunocytochemistry controls.

Cloning and transfection of hemagglutinin (HA)-tagged Ags

The pCI-neo vector (Promega) was modified to create a vector suitable for expression of N-terminal tagged type I transmembrane proteins with a fluorescent reporter under control of an internal ribosome entry site (IRES). Oligonucleotides were used to link the H2-Kb signal peptide and HA epitope tag (YPYDVPDYASL) to a unique multiple cloning site containing XcmI, Bsa36I, EspI, and XhoI recognition sequences, which was inserted at the Nhel-XbaI restriction sites of pCI-neo. IRES and ZsGreen sequences were amplified from the pHAGE-CMV-fullEF1a-IRES-ZsGreen vector, as created by J.-S. Lee provided by the Dana-Farber/Harvard Cancer Center DNA Resource Core (Boston, MA) and inserted at a NotI site downstream of the pCI-neo multiple cloning site.

Human myelin-associated glycoprotein (MAG) and oligodendrocyte-myelin glycoprotein (OMG) cDNA were obtained from Geneservice (www.geneservice.co.uk) and used to clone the extracellular, transmembrane, and cytoplasmic domains of each Ag by PCR using Phusion polymerase (New England Biolabs). These sequences, minus the endogenous signal peptide, were cloned into the modified vector with XcmI and XhoI enzymes (New England Biolabs). Jurkat cells were transfected by electroporation, and stable transfectants selected with 2 mg/ml G418. Cells expressing ZsGreen were sorted and stained with a biotinylated Ab to the HA tag (clone 3F10; Roche) and streptavidin-PE (Invitrogen). Single PE

Results

Detection of high-titer autoantibodies to native MOG in pediatric MS patients

Our previous FACS experiments used a MOG-GFP construct in which GFP was fused to the cytoplasmic domain of full length human MOG so that the brightness of GFP fluorescence reported on the level of MOG expression. A stably transfected Jurkat cell line had been generated, containing cells with different levels of MOG-GFP expression. FACS analysis of serum samples from ADEM patients showed a diagonal staining pattern for IgG binding vs GFP fluorescence (31), indicating that the labeling intensity closely correlated with the density of the Ag at the cell surface. This finding suggested that the sensitivity of the assay could be improved by single cell cloning of the cells that expressed the highest level of MOG-GFP. We therefore sorted single cells based on GFP fluorescence from MOG-GFP or control GFP transfectants and selected MOG-GFP (used as MOG throughout study) and GFP control clones with similar GFP expression levels for our experiments.

To enable sensitive detection of autoantibodies, a three-step staining procedure was used in which 50,000 MOG-GFP or control GFP cells were incubated with serum at a 1/50 dilution and bound Abs were detected using biotinylated anti-human IgG and streptavidin-PE. For each sample, the ratio of the MFI for the MOG-GFP and the control transfectant is shown (top left corner). The six depicted examples represent the entire range of fluorescent intensities for positive sera, with binding ratios of ~5–200 for the MOG vs the control transfectant. Ab titers measured by FACS analysis. Sera were incubated with MOG-GFP cells at the indicated dilutions. Abs to MOG were detectable in all positive sera at a dilution of ~1/400 and many at a dilution of 1/800.

FIGURE 1. Identification of autoantibodies to native MOG in pediatric MS patients. A, Brightness of fluorescent labeling with different positive pediatric MS sera. FACS data representing Ab binding to the MOG-GFP transfectant (open histograms) and the control GFP transfectant (shaded histograms) are shown for sera from six pediatric MS patients. Sera were incubated at a dilution of 1/50 with cells, and bound Abs were detected using biotinylated anti-human IgG and streptavidin-PE. For each sample, the ratio of the MFI for the MOG-GFP and the control transfectant is shown (top left corner). The six depicted examples represent the entire range of fluorescent intensities for positive sera, with binding ratios of ~5–200 for the MOG vs the control transfectant. B, Ab titers measured by FACS analysis. Sera were incubated with MOG-GFP cells at the indicated dilutions. Abs to MOG were detectable in all positive sera at a dilution of ~1/400 and many at a dilution of 1/800.

The online version of this article contains supplemental material.
MS sera. All positive sera labeled the MOG transfectant at a 1/400 dilution and several sera even at a 1/800 dilution (Fig. 1B). Comparison of the MOG-GFP and control GFP clones for each sample was important because the sera differed in the level of nonspecific binding to the Jurkat cells (Fig. 1A). The MOG clone gave a higher fluorescence signal than the MOG line and binding ratios that were typically 5- to 10-fold higher than with the lines (see supplemental Fig. 2). Because of their superior properties, these GFP and MOG clones were used in all subsequent analyses. Compared with the tetramer radioimmunoassay, the major advantages of this flow cytometric assay are that it enables higher throughput and avoids the use of radioactivity.

**MOG Abs define a subset of pediatric MS patients**

Sera from patients with pediatric- or adult-onset MS as well as control donors from an international group of clinics (Table I) were tested for Abs to MOG. Types of MS include relapsing-remitting (RRMS), secondary progressive (SPMS), and primary progressive (PPMS). Sera from patients with pediatric-onset MS patients, and infrequent in other inflammatory demyelinating CNS diseases. A. Sera from pediatric (left) or adult individuals (right) were assayed for Abs to MOG at a dilution of 1/50. The MOG to control binding ratio for each sample was calculated as described, and ratios greater than 5 were considered positive. Abs to MOG were significantly more common in pediatric- than adult-onset MS patients (p = 4.48 × 10⁻⁷) or control subjects and labeled the transfectant more brightly. B. Analysis of MOG Abs in patient populations from MS centers in different countries. Although anti-MOG was detectable in pediatric MS samples from all countries studied, the frequency of MOG Abs in adults varied considerably between centers.

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**Table I. Demographics of all pediatric- or adult-onset MS patient groups**

<table>
<thead>
<tr>
<th>Type of Sample</th>
<th>N</th>
<th>Percentage (%)</th>
<th>Age at Sample (y)</th>
<th>MS Subtype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Median</td>
<td>Range</td>
</tr>
<tr>
<td>Pediatric MS</td>
<td>131</td>
<td>63.3</td>
<td>14.6</td>
<td>5.0–19.4</td>
</tr>
<tr>
<td>Pediatric neurological control</td>
<td>34</td>
<td>55.9</td>
<td>13.7</td>
<td>2.1–18.5</td>
</tr>
<tr>
<td>Pediatric non-neurological control</td>
<td>37</td>
<td>67.6</td>
<td>12.3</td>
<td>2.0–16.0</td>
</tr>
<tr>
<td>Juvenile diabetes</td>
<td>28</td>
<td>71.4</td>
<td>10.7</td>
<td>5.7–17.6</td>
</tr>
<tr>
<td>Adult-onset MS</td>
<td>254</td>
<td>67.9</td>
<td>42</td>
<td>18–76.9</td>
</tr>
<tr>
<td>Adult control</td>
<td>86</td>
<td>56.5</td>
<td>40</td>
<td>22–64</td>
</tr>
<tr>
<td>Clinically isolated syndrome</td>
<td>30</td>
<td>80.0</td>
<td>37.9</td>
<td>22.9–54.3</td>
</tr>
<tr>
<td>Neuromyelitis optica</td>
<td>13</td>
<td>84.6</td>
<td>41</td>
<td>24–55</td>
</tr>
<tr>
<td>Viral encephalitis</td>
<td>29</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

* Sera from pediatric- or adult-onset MS donors as well as control donors from an international group of clinics were tested for Abs to MOG. Types of MS include relapsing-remitting (RRMS), secondary progressive (SPMS), and primary progressive (PPMS).

Data not available (n/a).
non-neurological diseases (n = 37) were negative. Among patients with other neurological diseases (n = 34), one (with a developmental delay) was clearly positive, whereas the other was marginally positive.

By comparison, MOG Abs were only detected in a small subset of adult-onset MS patients (11/254 sera, 4.3%) and the brightness of labeling with these serum samples was substantially lower than with the positive pediatric MS samples. The difference in anti-MOG frequency between all adult- and pediatric-onset MS cases was statistically significant (p = 4.48 × 10^-7). All anti-MOG-positive pediatric MS sera were from individuals with relapsing-remitting MS, the most common MS subtype in our pediatric group (Table I). Abs to MOG were found in all major clinical subtypes of adult-onset MS, including 6/171 relapsing-remitting, 3/49 primary progressive, and 2/34 secondary progressive patients. Abs to MOG were not detected in adults with NMO or viral encephalitis, and were rarely found at low levels in adults with a CIS (Fig. 2A). We have also initiated a prospective analysis of MOG Abs in pediatric demyelinating diseases to examine persistence of such Abs. As part of this ongoing study, we obtained serial serum samples from 39 children with demyelinating events, 5 of whom were positive for Abs to MOG. Serial samples were available in four of the five cases, and serum anti-MOG was detectable at each follow-up interval. Three patients had two samples each, taken at the first clinic visit and 4, 6, or 13 mo later. One patient had Abs to MOG in each of n = 3 serial samples, the last taken 33 mo after the initial visit and more than 4 years after the first clinical event of ADEM.

Matched serum and CSF samples were available from 13 pediatric patients; difficulties in obtaining CSF prevented analysis of more pediatric MS samples. Abs to MOG were detectable in the CSF of one pediatric-onset MS patient (binding ratio of 8.63 for CSF, ratio of 23.7 for paired serum) who had a first clinical event at 13.3 years of age. In two other pediatric MS cases with paired samples, MOG-specific Abs were detectable in the serum (binding ratios of 20.4 for CSF and 5.8 for paired serum) but not in paired CSF. It is important to consider that the IgG concentration is ~1000-fold higher in the serum compared with the CSF and that detection in the serum may therefore be more sensitive. Also, it is possible that Abs in CSF are present as immune complexes and therefore not able to bind MOG in our assay or that a fraction of MOG Abs are absorbed by their target Ag on oligodendrocytes/myelin.

The pediatric MS samples came from medical centers for MS in six different countries, and MOG-positive cases were identified for each center (Fig. 2B), with some differences in the frequency of positive cases. Among adult-onset MS patients, the frequency of Abs to MOG differed between centers (0–8.3% of positive sera). Although 12 of 43 pediatric MS samples from Canada contained anti-MOG, none of the 53 Canadian adult MS samples were MOG-positive (Fig. 2B). Because all these samples were analyzed by one individual using the same assay, differences in the frequency of MOG-positive adult-onset MS cases reported by different groups may reflect differences in the studied patient populations.

**MOG Abs are most prevalent in patients with a very early disease onset**

A number of possible factors may be responsible for the presence of MOG Abs in a subset of pediatric MS patients. No significant association between anti-MOG and gender or ethnicity was found (see supplemental Table I). However, the presence of Abs significantly correlated with a younger age at disease onset (p = 0.009): 38.7% of individuals with a first clinical event before age 10 had MOG Abs, compared with 14.7% of patients with a disease onset between 10 to 18 years (Fig. 3A). As stated above, the frequency of MS patients with MOG Abs was even lower in the adult-onset population (4.3%). In adult-onset MS, 3 of the 6 MOG-positive patients for whom data were available had their first clinical event at an age of over 40 years (Fig. 3B), and there was no correlation between the presence of these Abs and age at first clinical event (p = 0.1835). Abs to MOG were found in pediatric MS patients with recent disease onset (<1 year) and also those with a longer disease course (>5 years) (Fig. 3C), indicating that MOG can be a target in early stages of MS, and that the Abs may persist, at least for some time. Most patients were either untreated or...
had received IFNs at the time of sample collection (see supplemental Table II), and the usage of specific immunomodulatory drugs was not significantly different in the MOG-positive and -negative groups ($p = 0.636904$).

Based on our previous findings of MOG Abs in ADEM and the fact that this disease is most common in children, we investigated the relationship between Abs to MOG and clinical presentation at the first demyelinating event. A significant association was found between anti-MOG and a first clinical event diagnosed as ADEM ($p = 0.0049$): 30.8% of MOG-positive MS patients had an initial diagnosis of ADEM, compared with 8% of MOG-negative cases. Also, 50% of pediatric MS cases with an initial ADEM-like first event had MOG Abs (see supplemental Table I).

Functional properties of MOG Abs from pediatric MS patients

Abs to MOG may cause demyelination and oligodendrocyte death by either complement activation or Fc receptor-dependent mechanisms. IgG subtypes differ in the ability to fix complement and induce Ab-dependent cellular cytotoxicity, and we therefore subclassified MOG-specific IgG Abs by FACS using a panel of secondary Abs specific for defined IgG subtypes (IgG1-IgG4). The majority of sera contained only MOG-specific IgG1 (7 of 12 sera), one sample had both MOG-specific IgG1 and IgG3 and another was dominated by IgG2 (Fig. 4). The lower sensitivity of the subclass-specific secondary Abs compared with the pan-IgG Ab prevented identification of the dominant IgG subtype in three samples. Human IgG1 is efficient in fixing complement and inducing Ab-dependent cell-mediated cytotoxicity.

The murine 8-18C5 mAb to MOG is well known to bind oligodendrocytes in culture and induce demyelination in vivo. We tested whether Abs from pediatric MS patients could compete with 8-18C5 for binding to MOG-GFP cells. Because the secondary Ab used to detect bound IgG recognizes human but not mouse proteins, this assay was not confounded by detection of 8-18C5 on the cells. Addition of 8-18C5 decreased the binding of patient serum Abs to MOG-GFP cells in a dose-dependent manner for all samples tested (Fig. 5A). This result shows that the Abs from pediatric MS patients bind to surfaces on MOG that significantly overlap with the 8-18C5 epitope.

To further verify the specificity of these Abs, we affinity purified polyclonal Abs from sera using a MOG protein preparation representing the extracellular domain that had been refolded from E. coli inclusion bodies. The protein carried a C-terminal BirA tag for site-specific biotinylation, enabling capture onto streptavidin beads. The MOG extracellular domain contains a single Ig domain and we used a protein of similar size (membrane proximal Ig domain of CD80) as a control. Biotinylated MOG or CD80 were bound to streptavidin-coated agarose beads for affinity isolation of MOG-specific Abs from 5 μl of serum. Unbound and eluted Abs were used to stain MOG and control transfectants. Binding ratios obtained with purified Abs from four sera are summarized in the table. Abs purified on MOG beads bound MOG on the cell surface, and addition of 25 μg of soluble recombinant MOG inhibited binding of eluted MOG-specific Abs for all four sera tested.

**FIGURE 4.** The majority of pediatric MS patients have MOG IgG1 Abs, an IgG subtype that can fix complement and bind to Fc receptors. IgG subclass-specific secondary Abs were used to detect the binding of serum Abs to the MOG transfectant. In the example shown, only MOG-specific IgG1 Abs were detected. IgG1 was the most common subtype among these pediatric MS patients, found in 8 of 12 tested sera. IgG2 and IgG3 were rarely found, and IgG4 was not detected in any sample.

**FIGURE 5.** Abs in pediatric MS sera are specific for MOG. A. A mAb to MOG competes with serum Abs for Ag binding. Sera and indicated amounts of the MOG-specific Ab 8-18C5 were incubated together with MOG-GFP cells for 1 h. A human-specific secondary Ab was used to detect bound IgG. Addition of increasing amounts of 8-18C5 decreased the amount of serum Ab bound to cells. B. Affinity-purified Abs specifically bind to MOG transfectants. Biotinylated recombinant MOG (rMOG, extracellular domain) or an Ig superfamily control protein (membrane-proximal Ig domain of CD80) were captured onto streptavidin beads for affinity isolation of MOG-specific Abs from 5 μl of serum. Unbound and eluted Abs were used to stain MOG and control transfectants. Binding ratios obtained with purified Abs from four sera are summarized in the table. Abs purified on MOG beads bound MOG on the cell surface, and addition of 25 μg of soluble recombinant MOG inhibited binding of eluted MOG-specific Abs for all four sera tested.
binding was not glycosylated, indicating that at least a subset of MOG-specific Abs in pediatric MS sera do not require the N-linked glycan for binding. These experiments firmly establish that the Abs detected with our flow cytometric assay are indeed specific for MOG.

MOG-specific Abs label human white matter and myelinated axons

We next sought to determine whether MOG-specific Abs from pediatric MS patients can bind MOG in the CNS and therefore performed immunocytochemistry on acetone-fixed human brain sections containing both white and gray matter using biotinylated total serum IgG was purified on MOG or BSA-coated beads, and affinity purified Ab corresponding to 100 μg of total IgG was used per stain. MOG-specific Abs from pediatric MS patients W52 and W24 labeled white matter in a myelin and oligodendrocyte-like pattern. No staining was seen with Abs isolated on BSA control beads or Abs from a control donor (D14). Scale bar represents 25 microns. C, MOG-specific Abs label myelinated axons and satellite glia in the subpial gray matter. Regions of gray matter in the same tissue sections as shown above were assessed for Ab binding. Small bundles of myelinated axons were labeled by the MOG mAb 8-18C5 and total IgG from a MOG-positive ADEM patient (R4), and satellite glia cell bodies were identified with Ab 14E (data not shown). Pediatric MS serum Abs (patient W52) purified on MOG but not BSA beads also bound myelinated axons. Scale bar represents 50 microns.

This observation was confirmed by immunostaining of acetone-fixed human brain sections with Abs affinity purified on MOG and BSA-coated beads (Fig. 6B). MOG-specific Abs from pediatric MS patients W52 and W24 labeled white matter in a myelin and oligodendrocyte-like pattern. No staining was seen with Abs isolated on BSA control beads or Abs from a control donor (D14). Scale bar represents 25 microns. C, MOG-specific Abs label myelinated axons and satellite glia in the subpial gray matter. Regions of gray matter in the same tissue sections as shown above were assessed for Ab binding. Small bundles of myelinated axons were labeled by the MOG mAb 8-18C5 and total IgG from a MOG-positive ADEM patient (R4), and satellite glia cell bodies were identified with Ab 14E (data not shown). Pediatric MS serum Abs (patient W52) purified on MOG but not BSA beads also bound myelinated axons. Scale bar represents 50 microns.

Total IgG was isolated from anti-MOG positive and negative sera, biotinylated and then further purified using beads with immobilized MOG or BSA as a control protein. In samples from two MOG-positive pediatric MS cases (W52 and W24), Abs affinity purified on MOG beads but not control BSA beads labeled CNS white matter, whereas no staining was observed with affinity purified Abs from control donor D14 (Fig. 6B). Abs from patient W52, and more weakly with W24, also labeled cell bodies in the white matter similar in morphology to cells identified with the oligodendrocyte-specific Ab 14E and other oligodendrocyte-specific Abs.

Myelinated axon bundles in the subpial cortical gray matter were stained with the anti-MOG mAb 8-18C5 and serum IgG from a previously studied ADEM patient (R4) with high-titer MOG Abs (Fig. 6A) (and see supplemental Fig. 3A,4) and streptavidin-PE. C, Pediatric MS sera with MOG Abs do not show broad anti-myelin reactivity. Ab binding for 13 serum samples is shown as the MFI of PE. Although Abs to MAG and OMG were detectable at low levels in a few sera, the labeling of MOG transfectants was considerably brighter in all cases. Abs to OMG and MAG were not detected in MOG-negative pediatric MS sera or pediatric controls (data not shown).

We also performed staining with total biotinylated serum IgG not purified on MOG or BSA beads (see supplemental Fig. 3).4 IgG from ADEM patient R4 stained white matter in a myelin-like pattern (see supplemental Fig. 3B).4 IgG from pediatric MS patient W52 labeled myelin and glial cells, whereas IgG from pediatric
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conflicting results. Although some found Abs to MOG in patients unknown. of the involved B cells and their Ab products remain largely the pathogenesis of MS remains unresolved. Also, the specificities elimination could substantially decrease T cell priming and acti-

Ag-specific B cells are highly effective as APCs (39, 40), and their inflammation through production of cytokines and chemokines. present myelin-derived Ags to autoreactive T cells and driving progression not only as a source of autoantibodies, but also by present in a substantial subset of pediatric-onset MS patients, in particular children with a very early disease onset. Stringent criteria establish their Ag specificity: the Abs label MOG but not control transfectants, they can be affinity purified with recombinant MOG but not a structurally related control protein, and addition of soluble MOG inhibits Ab binding. Abs to MOG from pediatric-onset MS patients and 25 pediatric controls did not contain Abs to OMG or MAG (data not shown). MOG is therefore a more frequent target for autoantibodies in pediatric MS than MAG or OMG.

Discussion

These results show that circulating high-titer Abs to MOG are present in a substantial subset of pediatric-onset MS patients, in particular children with a very early disease onset. Stringent criteria establish their Ag specificity: the Abs label MOG but not control transfectants, they can be affinity purified with recombinant MOG but not a structurally related control protein, and addition of soluble MOG inhibits Ab binding. Abs to MOG from pediatric-onset MS patients also bound myelin in normal human white matter and myelinated axons in subpial gray matter. The majority of MOG-specific Abs have the IgG1 subtype that can fix complement and bind to Fc receptors, indicating that they have the potential to damage myelin or oligodendrocytes if they gain access to the CNS. Such Abs were rarely detected in pediatric controls or patients with adult-onset demyelinating conditions, including MS. In the pediatric patient population, their presence strongly correlated with age at disease onset and an initial ADEM-like presentation, but not with gender or ethnicity. Future prospective studies will examine the relationship between such an Ab response and clinical disease course, MRI features, response to treatment, and prognosis.

The pronounced therapeutic response to B cell depletion with rituximab shows that B cells play a central role in the pathogenesis of adult-onset MS. It is likely that B cells contribute to disease progression not only as a source of autoantibodies, but also by presenting myelin-derived Ags to autoreactive T cells and driving inflammation through production of cytokines and chemokines. Ag-specific B cells are highly effective as APCs (39, 40), and their elimination could substantially decrease T cell priming and activation. The relative importance of these different mechanisms to the pathogenesis of MS remains unresolved. Also, the specificities of the involved B cells and their Ab products remain largely unknown.

Discordant studies of autoantibodies in adult-onset MS yielded conflicting results. Although some found Abs to MOG in patients with MS or CIS (41, 42), others reported no difference in the frequency of anti-MOG between MS and other neurological diseases (43–45) or healthy controls (46). This controversy surrounding the presence of MOG Abs in MS patients is likely attributable to differences in detection methods. Our flow cytometric approach therefore emphasized detection of Abs to native, properly folded MOG protein.

Three other groups also generated MOG-expressing cells to examine the presence of autoantibodies in adult-onset MS patients. Haase et al. (47) generated a stable MOG transfectant in LTK− cells and found that only one of 17 serum samples from adult MS patients labeled this transfectant, even though all patients as well as healthy control subjects had Abs that detected linear MOG peptides in an ELISA. Lavile et al. compared serum Ab staining of a MOG transfectant Chinese hamster ovary cell line to nontransfected Chinese hamster ovary cells and concluded that IgG Abs specific for native MOG were most frequently found in the serum of patients with CIS and relapsing-remitting MS (48). However, the binding ratios of the MOG and control cells were less than two for all positive samples, even though high serum concentrations were used for staining (1/10 dilution, compared with the 1/50 dilution used in this study). We defined binding ratios over 5 as positive, and obtained binding ratios as high as 200.2 in pediatric MS samples.

In another study, Zhou et al. (49) transduced a human glioblastoma cell line with a lentivirus encoding MOG and stained these cells at a serum dilution of 1/36. The MOG-specific Ab response was calculated by subtracting median fluorescence intensity values obtained with MOG and control transfectants. The authors reported that 32% of adult-onset MS patients and 4% of control subjects had detectable Abs, but the difference in MFI between the MOG and control cells was rather small (<50) for most sera. By comparison, we observed differences in MFI of 516 to 22,102 for the six representative examples shown in Fig. 1A. We prefer to express the data as a binding ratio between the MOG and control transfectants rather than as a difference in MFI because the level of background differs greatly between sera. For example, a sample with a high background and a small MOG-specific increase in binding can have a large change in MFI (for example, MFI values of 600 and 500 for MOG and GFP transfectants, respectively), even though the binding ratio is small (1.2 in this example). Although both analysis methods permit the identification of samples with high levels of MOG-specific Abs, using MFI differences can result in the classification of samples with a high background as positive.

In this study, we have analyzed the largest collection of adult-onset MS sera to date (n = 254), and the results show differences in the frequency of MOG-positive cases between samples from MS centers in Canada (0/53, 0%), the US (5/129, 3.9%), and Switzerland (6/72, 8.3%). These apparent differences may be caused by the overall low frequency of Abs to MOG in adult-onset MS, but differences in patient selection or genetic and environmental factors cannot be excluded. Sample handling may also be a contributing factor, but appears unlikely because we observed that MOG Abs remain detectable after multiple freeze-thaw cycles.

Our findings support the conclusion that Abs to MOG are rather uncommon among adult-onset MS patients, whereas the anti-MOG reactivity identified in a subset of pediatric MS cases is among the strongest autoantibody responses observed so far in MS. Using multimeric forms of folded and glycosylated MOG protein, we previously detected similar Abs to MOG in a subset of ADEM patients (31). In the current study of pediatric MS, the presence of MOG Abs strongly correlated with an initial ADEM-like onset, although all children subsequently experienced two or more non- ADEM demyelinating events as required for a diagnosis of MS (19). The 50% of pediatric MS patients with an initial diagnosis of
ADEM had Abs to MOG, whereas 92% of MOG negative pediatric MS patients did not have an ADEM-like initial event. Prospective studies are required to further define the relationship between MOG Abs and ADEM as well as pediatric MS.

Why have we only detected Abs to MOG, but not MAG or OMG, which are also myelin surface Ags with large extracellular domains? Chronic inflammation can result in immune responses to multiple self-Ags, a phenomenon referred to as epitope spreading (50). However, significant immune responses are not mounted to every Ag in the target structure. MOG is a highly encephalitogenic protein in immunization-based animal models of MS and is the only known myelin component to induce pathogenic Ab and T cell responses (51, 52). MOG also has substantial sequence similarity with the milk protein butyrophilin, and Ab as well as T cell cross-reactivity between these Ags has been demonstrated (53, 54).

Why does the prevalence of these autoantibodies change with age at disease onset in pediatric populations? Several general and possibly interrelated factors may be involved: genetic susceptibility, the kinetics and magnitude of the autoimmune response, environmental factors, and the biology of myelination during childhood. In type I diabetes, children with particular combinations of MHC class II genes are more likely to become diabetic at a young age and thus appear to carry a higher genetic risk (55). Similarly, children who develop MS at a young age may carry combinations of genes that raise susceptibility to MS to a higher level than in individuals who develop MS later in life. The frequency and functionality of MOG-specific T cells could also affect production of MOG Abs. The importance of T cell–B cell collaboration was highlighted by recent studies which showed that genetically engineered mice with a high frequency of both MOG-specific T cells and B cells spontaneously develop severe CNS inflammation, whereas mice with only a high frequency of MOG-specific B cells remain healthy (56–58).

This study shows that MOG Abs identify a subset of pediatric MS patients with a very early disease onset. In contrast, MOG Abs are less common in patients with adult-onset disease. Future studies will address the pathogenetic significance of the emergence of such autoantibodies in pediatric demyelinating diseases.

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References


