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Anti-myelin antibodies modulate clinical expression of childhood multiple sclerosis

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ABSTRACT

Anti-myelin basic protein (MBP) antibodies in pediatric-onset MS and controls were characterized. Serum samples were obtained from 94 children with MS and 106 controls. Paired CSF and serum were obtained from 25 children with MS at time of their initial episode of acute demyelinating syndrome (ADS). Complementary assays were applied across samples to evaluate the presence, and the physical binding properties, of anti-MBP antibodies. While the prevalence and titers of serum anti-MBP antibodies against both immature and mature forms of MBP were similar in children with MS and in controls, binding characteristics and formal Surface Plasmon Resonance (SPR) studies indicated surprisingly high binding affinities of all pediatric anti-MBP antibodies. Serum levels of anti-MBP antibodies correlated significantly with their CSF levels, and their presence in children with MS was associated with significantly increased risk of an acute disseminated encephalomyelitis-like initial clinical presentation. While antibodies to both immature and mature forms of MBP can be present as part of the normal pediatric humoral repertoire, these anti-myelin antibodies are of surprisingly high affinity, can access the CNS during inflammation, and have the capacity to modulate disease expression. Our findings identify an immune mechanism that could contribute to the observed heterogeneity in spectrum of clinical presentations in early-onset MS.

Abbreviations: ADEM, acute disseminated encephalomyelitis; RU, resonance unit; k_a, association rate constant; k_d, dissociation rate constant; K_D, equilibrium dissociation constant; M, mol/L.

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1. Introduction

Multiple sclerosis (MS) involves early life triggering of abnormal immune responses to particular CNS self-antigens in hosts whose genetic predisposition may reflect both immune system and target-organ susceptibilities (Bar-Or, 2008; Prat and Antel, 2005). Initiating processes and earliest targets of both cellular and humoral immune responses have not been elucidated. Pathology studies indicate that actively demyelinating MS lesions often involve deposition of immunoglobulin (Ig) and complement Lucchinetti et al. (2000). Understanding the contribution of anti-myelin antibodies to early disease events is increasingly relevant as the field shifts towards earlier intervention and, in particular, as B cell-directed therapies are demonstrating promise in MS clinical trials (Bar-Or et al., 2008; Giacomini et al., 2009; Hauser et al., 2008).

A challenge in studies of adult-onset MS is that an initial clinical presentation has likely been preceded by years of sub-clinical biological disease activity, making it difficult to evaluate whether antibodies to particular myelin epitopes participate in injury, or are merely generated as a consequence of such injury. By comparison, studying pediatric-onset MS provides a unique opportunity to evaluate putative disease targets and early events, closer to the biological onset (Banwell et al., 2007a,b). This has been recently highlighted by the identification of myelin oligodendrocyte glycoprotein (MOG)-reactive autoantibodies in children with MS and acute disseminated encephalomyelitis (ADEM), but not in adults (Brilot et al., 2009; McLaughlin et al., 2009). Interestingly, prospective studies now indicate that up to 20% of children with MS experienced an initial CNS demyelinating episode that met all clinical criteria for the diagnosis of ADEM (multifocal CNS involvement and presence of encephalopathy, with or without additional features such as fever or meningismus) (Banwell et al., 2007b; Krupp et al 2007).

An additional consideration in pediatric-onset disease, is that CNS myelin continues to develop in the early life, such that the molecular composition of potential antigenic targets changes with myelin maturation. This may be particularly relevant for myelin basic protein (MBP), which in the pediatric (immature) form contains post-translational modifications that are not present in normal adult (mature) MBP, yet may be disease relevant (Kim et al., 2003; Mastronardi and Moscarello, 2005; Wood and Moscarello, 1989). For example, pediatric-derived MBP contains a skewed distribution of charge isomers, not found in the normal adult brain, but very similar to that reported in adult MS lesions (Wood et al., 1996). Immature MBP is also highly citrullinated compared to adult MBP, and increased citrullination of proteins has been known to enhance their immunogenicity in the context of autoantibodies and rheumatoid arthritis (Klareskog et al., 2008; van Venrooij and Pruijn, 2000) as well as in mouse models of both autoimmune arthritis and CNS demyelination (Kidd et al., 2008).

Here, using a series of complementary techniques, we investigated the presence and the binding characteristics of serum antibodies to both the mature and immature forms of human-derived MBP, in a large cohort of clinically well-characterized children with MS and controls. Our study provides novel insights into the potential contribution of anti-myelin antibodies in the early MS disease process.

2. Materials and methods

2.1. Participants

Children diagnosed with MS using Poser criteria (McDonald et al., 2001; Poser et al., 1983) and healthy or 'other disease' controls, all prior to age 17 years 11 months and cared for in the 17 participating institutions (located in Canada, the United States, Argentina, Russia, Italy, and Finland) were eligible for inclusion. All children were examined on the day of serum collection, and detailed demographic and clinical data were obtained by standardized clinical interview of children and parents, subsequently substantiated by medical record review. Physical examination findings and an expanded disability status score (EDSS) (Kurtzke, 1983) were recorded for each child with MS. In addition, matched serum and CSF samples were obtained from 25 children with a first demyelinating event (termed, acute demyelinating syndrome, ADS), who were subsequently monitored prospectively for the development of MS. For the subgroup of children with MS whose first demyelinating event met clinical criteria for acute disseminated encephalomyelitis (ADEM), establishing the diagnosis of MS required each child to experience two or more subsequent non-ADEM like demyelinating attacks (Krupp et al., 2007). Informed consent and assent were obtained from all participants. The study was approved by the Research Ethics Departments of all participating institutions.

2.2. Antigens and immunoassays

MBP was isolated as previously described (Cheifetz and Moscarello, 1985), from normal adult and pediatric (four-month-old female) human brains. All assays utilized identical specimen aliquots, blindly distributed from a centralized sample repository, and MBP derived from the identical preparations. Anti-MBP immunoblotting was performed according to standard procedures. Briefly, MBP (10 μg per lane) was resolved by 12% SDS-PAGE then transferred onto nitrocellulose membranes. Blocked (5% milk in neutral buffer containing 0.5% Triton X-100) blots were cut into strips and probed sequentially with sera (1:500) and HRP-conjugated anti-human IgG/A/M (Chemicon) diluted in blocking solution. Strips used for positive controls were sequentially probed with polyclonal goat anti-MBP (Syn. X Pharma Inc.; Toronto, ON, Canada) and HRP-conjugated anti-sheep/goat IgG (Chemicon). Bound antibodies were visualized using chemiluminescence (Amersham). Immunoblot images were blindly assessed and the highly purified C1 MBP isomer (isolated as described (Chou et al., 1976)) was used to confirm specificity of serum binding to the immature and mature whole MBP preparations.

In ELISA assays, serum (1:80) was added to wells coated with 250 ng of MBP or control antigen. Bound antibodies were detected as described (O'Connor et al., 2003) using goat anti-human IgG Fcγ fragment specific HRP-conjugated antibodies (1:15,000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) or IgM(Fc)-HRP-conjugated antibodies (Jackson) for detection. For direct binding and serial dilution studies by DELFIA (dissociation-enhanced lanthanide fluoro-immunoassay), serum and CSF were diluted 1:80 or 1:10, respectively, then applied to wells coated with 250 ng of either MBP or control antigen. Bound antibodies were detected using a strategy similar to one previously described (O'Connor et al., 2005); in the solution-phase format, serum (1:80) was incubated with 5 μg/ml of soluble antigen for 5–6 h at 4 °C, then added to previously coated and blocked plates. Bound antibodies were detected as described above. Subclasses of IgG that bound MBP were determined using the DELFIA as described above with individual IgG subclass specific secondary antibodies (IgG_1 and IgG_2 from Invitrogen Carlsbad, CA, IgG_3 from Pierce/Thermo, Rockford, IL and IgG_4 from Zymed, San Francisco, CA).

2.3. Mass spectrometry

The molecular mass of pediatric-derived MBP isoforms was obtained on QStAR XL electrospray ionization QToF mass spectrometer (Applied Biosystems/MDS SCIEX, Concord, Ontario, Canada) in positive ion mode. Samples (5 μg) were dissolved in a solution of acetonitrile and deionized water (1:1 V/V) containing 0.2% formic acid. Samples (2 μl) were applied to a reverse-phase capillary column using an HPLC system (Waters, Milford, MA, USA) then carried over to a nanospray source by a mobile phase of 50% acetonitrile in deionized water containing 0.2% formic acid. The flow rate was kept at 6 μl/min and the capillary voltage was maintained at 3000 eV. The resulting

mass spectra were deconvoluted using the Bayesian Protein reconstruct feature of the ABI Bioanalyst 1.1 software.

2.4. Surface Plasmon Resonance

Serum-derived IgG binding to immature and mature human MBP was examined at 25 °C using a Biacore 3000 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) with research-grade CM4 sensor-chips and HBS-EX buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.05% (v/v) Triton X-100). The high quality of pediatric and adult MBP preparations was verified by total amino acid analysis and mass spectrometry. IgG was isolated from the pediatric serum samples using Protein G HP Spin Trap columns (GE Healthcare). Low- or high-density MBP surfaces (2 μg/ml in 10 mM sodium acetate pH 5.5; 300–3000 resonance units (RU) final) were immobilized using the Biacore Amine Coupling Kit, with corresponding reference surfaces prepared in the absence of MBP. Active MBP surfaces and consistent replicate injections (30 μl/min; 3 min association + 5 min dissociation) were verified using commercially available anti-MBP polyclonal antibody (0–0.75 μM; Dako, California, USA). To screen for relative differences in MBP binding affinity, purified IgG samples (0–1.2 μM) and an internal anti-MBP (1/200) control were injected (5 μl/min; 15 min association + 15 min dissociation) and binding data doubled-referenced as previously described (Myszka, 1999). Due the inherent heterogeneity of the purified IgG samples (i.e. IgG_{1-4} subclasses) and pediatric/adult forms of MBP (i.e. C1–C8 isomers), the lowest 0.15 μM IgG binding profiles were compared using a simplified bivalent analyte model (Pol et al., 2007). Briefly, the R_{max} (theoretical binding maximum at saturating analyte concentration, RU) and k_t (mass transfer coefficient, RU/mol l^{-1} s) parameters were fit globally whereas k_a (apparent association rate constant, 1/mol l^{-1} s) and k_d (apparent dissociation rate constant, 1/s) were fit locally in order to determine the apparent equilibrium dissociation constants ($K_D = k_d/k_a$, mol/l).

2.5. Statistical analyses

The Chi squared test (for comparisons between proportions) or the Fisher exact test (non-parametric analyses) were applied for between group comparisons where indicated. Adjustments for multiple comparisons carried out for cross-group comparisons of data from Western blot, ELISA and DELFIA assays did not impact the results. The Spearman test was used to assess the correlation between CSF and

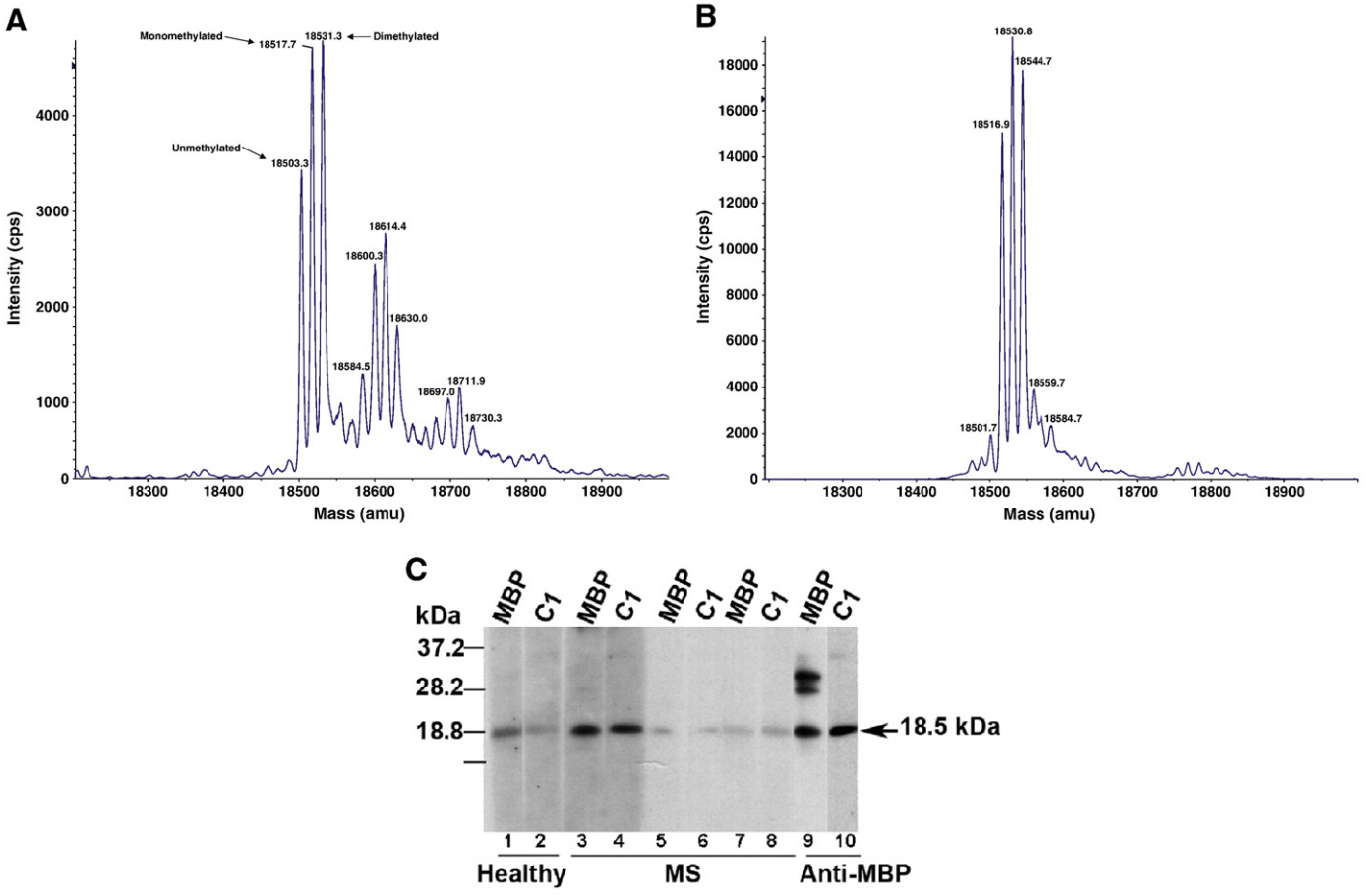

Fig. 1. Mass spectrometry of immature and mature MBP and examples of Western (immunoblot) detection of anti-MBP serum reactivities in pediatric-onset MS and healthy pediatric controls. Mass spectrogram of pediatric-derived MBP (MBPT2) showing a range of isoforms (A) not represented in the more mature adult-derived MBP (B). The three peaks shown between 1.850×10^4 and 1.855×10^4 represent the methylated (ARG 107) forms of MBP. The unmethylated is shown at 18,503.1992; the monomethylated at 18,517.6992 (14 atomic mass units greater than the unmethylated); and the dimethylated species at 18,531.3008 (28 mass units greater than the unmethylated species). MBP species of lower intensity are shown between 1.860×10^4 and 1.863×10^4. These represent methylated and phosphorylated species since they differ from the major species by 80 amu. The unmethylated, phosphorylated species has a mass of 18,585.6992 amu; the monomethylated, phosphorylated species has a mass of 18,600.3008 amu; the dimethylated, phosphorylated has a mass of 18,614.4004 amu. Immunoblot detection of anti-MBP serum reactivities in healthy pediatric control and pediatric-onset MS (C). Examples of serum reactivities are shown for a healthy control (strips 1 and 2) and three children with MS (strips 3 to 8). In each case, probing is done to both human MBP and the highly purified C1 isomer of human MBP (C1) as a specificity control. Positive control (monoclonal anti-MBP antibody; strips 9 and 10), low range molecular weight standards are shown at the left and the 18.5 kDa MBP isoform is indicated by an arrow at the right.

serum antibodies, with a value of $p \leq 0.05$ considered significant. The Mann–Whitney test was used to assess the difference between MS and control-derived MBP antibody equilibrium dissociation constants (K_D), with a value of $p \leq 0.05$ considered significant. Pearson's correlation assessed association between serum antibody levels and clinical characteristics (age at onset and total relapses) in patients diagnosed with MS.

3. Results

3.1. Immature versus mature human MBP

Direct comparison of pediatric- and adult-derived MBP by mass spectrometry revealed that, in addition to the previously reported (Wood and Moscarello, 1989) increased citrullination, the immature pediatric-derived MBP (Fig. 1A) harbored a number of modifications, including several prominent phosphorylated species not present in the more mature, adult-derived MBP (Fig. 1B).

3.2. Anti-MBP measures in pediatric MS and controls by immunoblot

Two hundred children (94 with MS and 106 controls) were recruited and their detailed demographic and clinical data are provided in Supplementary Table 1. Physical examination findings and EDSS scores for children with MS are provided in Supplementary Tables 1 and 2. Frequencies of serum immune reactivities to both the mature and immature forms of MBP were assessed in all 200 children using an immunoblot assay. To ascertain specificity, all samples deemed positive in an initial run were confirmed in subsequent immunoblotting using both the whole MBP preparations as well as the highly purified 18.5 kDa C1 isomer of MBP (Fig. 1C). Samples from 3 MS and 3 healthy controls could not be interpreted because of high background. In the remaining samples, serum reactivities to the mature form of MBP were detected in 22 of 91 (24%) children with MS (Table 1), which was not different from healthy controls (20%, $p = 0.9939$). Similarly, the frequencies in the 'other autoimmune disease' (32%) and 'other neurological disease' (19%) cohorts did not differ from healthy controls ($p = 0.2062$ and $p = 0.9939$ respectively). Serum reactivities to the immature form of MBP (Table 1) were detected in 17 of 91 (19%) children with MS, which was again no different than the reactivities to the immature MBP detected in the healthy control group ($p = 0.8417$).

3.3. ELISA and DELFIA

We used ELISA and DELFIA techniques to quantitatively compare anti-MBP antibodies in children with MS and controls. While ELISA has been used in several prior studies of anti-myelin antibodies in adult MS cohorts (Reindl et al., 1999; Vojdani et al., 2003), the fluorescence-based DELFIA is described as having greater sensitivity and a wider dynamic range than ELISA (Butcher et al., 2003). Both IgG and IgM anti-MBP antibodies were assessed, as were reactivities to histone H1—a protein very similar to MBP in terms of charge density and molecular weight. None of the serum samples exhibited IgG or IgM antibody responses to this control antigen that exceeded 15% of those observed with MBP, in either ELISA or DELFIA, supporting the specificity of our anti-MBP measurements. Whether using the predefined arbitrary cut-off of 2 s.d. above the mean of the healthy control cohort to define 'seropositivity' (dashed lines, Fig. 2), or less stringent cut-off criteria (such as 1.5 or 1.0 s.d.), we found no differences in the frequencies of anti-MBP antibody seropositivity, or in the average optical densities (ELISA) or mean europium counts (DELFIA) across the pediatric MS and control cohorts, for either IgG anti-MBP antibodies (Fig. 2A and B, respectively), or IgM-anti-MBP antibodies (Fig. 2C and D, respectively). We did observe strong correlations between the results obtained from the ELISA and DELFIA techniques for both IgG and IgM (Fig. 2E, F).

3.4. Further qualitative and quantitative examination of anti-MBP antibodies

The presence of antibodies to both immature and mature MBP in similar frequencies and levels in the serum of both children with MS and controls, indicates that anti-myelin antibodies can be present as part of the normal humoral immune repertoire in children. We next considered whether, when present, such anti-myelin antibodies might exhibit different binding characteristics across cohorts. We therefore investigated the physical properties of anti-MBP antibodies in representative samples of MS and control children identified as seropositive. Titers of 80 (80-fold serum dilution) or greater were confirmed in 92% of samples tested for IgG and 100% of samples tested for IgM (Table 2), and no differences were observed between MS and control sera for either IgG or IgM antibody titrations. Since antigens studied in solid phase assays may adopt a different conformation than their soluble form, we further studied the ability of serum antibodies to bind MBP in a competitive solution-phase assay. Of 12 representative samples (including both children with MS and pediatric controls) identified as harboring IgG anti-MBP antibodies, 8 (67%) were found to have antibodies that also bound MBP in solution (Table 2). Similarly, of 9 representative samples identified as harboring IgM anti-MBP antibodies, 5 (56%) also bound MBP in the solution phase. The specificity of binding was supported by the observation that soluble lysozyme did not inhibit serum binding to solid phase MBP for either IgG or IgM. No differences were observed between MS and controls for all solution binding experiments.

To directly quantify the binding affinities of anti-MBP antibodies in the pediatric sera (to either form of MBP), we next used label free, real-time Surface Plasmon Resonance (SPR) technology. The binding affinities of anti-MBP antibodies (Table 3) between control and MS samples were not statistically different ($p = 0.418$ and $p = 0.503$ for immature and mature MBP respectively). We were, however, surprised to observe very slow dissociation rates in all children that were consistent with high-affinity anti-MBP binding interactions. Indeed, the apparent rate constants predicted ($k_a = 10^3$ to $10^4\ \mathrm{mol^{-1}\,l\,s^{-1}}$; $k_d = 10^{-3}$ to $10^{-4}\ \mathrm{s^{-1}}$; $K_D = 10^{-8}$ to $10^{-10}\ \mathrm{mol\,l^{-1}}$) were similar to those observed for murine-derived anti-MBP, run in the same assay,

Table 1
Pediatric serum reactivities against mature and immature forms of MBP by immunoblot.

Cohort	Mature MBP			Immature MBP		
	Number	Anti-MBP positive (%)	*p* value	Number	Anti-MBP positive (%)	*p* value
MS	91	22 (24%)	0.9939	91	17 (19%)	0.8417
Other autoimmune	28	9 (32%)	0.2062	28	5 (18%)	0.7356
Neurological	26	5 (19%)	0.9939	26	5 (19%)	0.8430
Healthy	49	10 (20%)	–	49	9 (18%)	

Results are reported relative to the positive control to enable comparisons across blots. Samples with similar or greater reactivity than that of the control were considered positive. Proportions of positive reactivity for disease cohorts were compared to the healthy control cohort (Chi squared test for association); no significant differences were with *p* value adjusted for multiple comparisons. Three MS patients and three healthy donor samples were excluded from this analysis because the immunoblots were not interpretable.

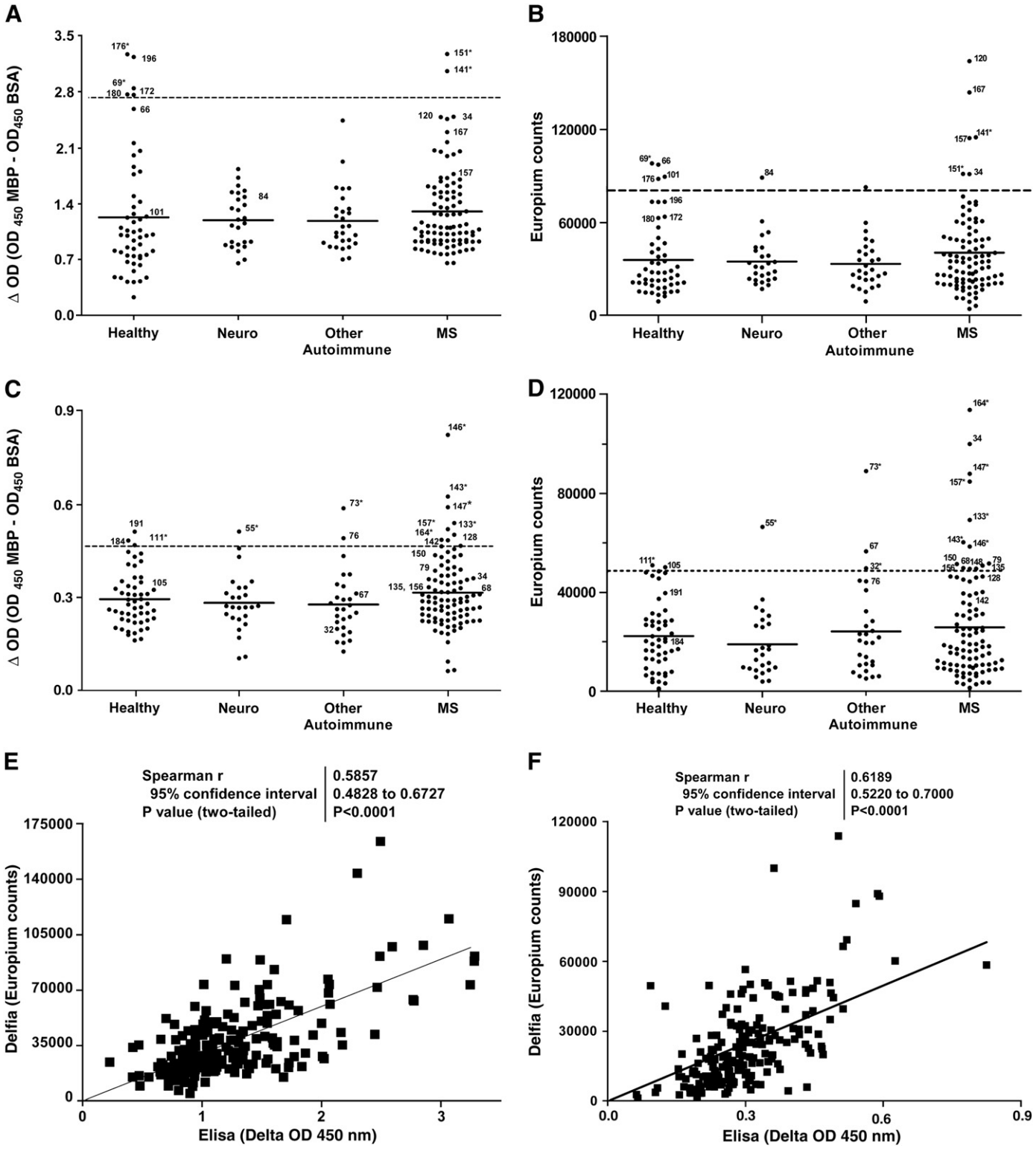

Fig. 2. ELISA and DELFIA detection of serum IgG and IgM anti-MBP antibodies. ELISA and DELFIA are shown for IgG anti-MBP antibodies (A and B, respectively) and for IgM anti-MBP antibodies (C and D, respectively). Results obtained from ELISA and DELFIA correlated well for both IgG (E) and IgM (F) anti-MBP antibodies. Numbered symbols identify particular samples for ease of comparison. Samples determined to be positive by both ELISA and DELFIA are indicated by an asterisk. ELISA results were reported as ΔOD ([OD450–OD540] of wells coated with MBP minus the [OD450–OD540] of wells coated with BSA). The mean ΔOD was normalized to a quality control serum sample. The dashed lines represent values that are 2 standard deviations (s.d.) above the mean of the healthy control samples.

and resemble the high affinities typically reported for human serum antibodies directed toward pathogens, such as the influenza virus (Wrammert et al., 2008).

Finally, the IgG subclass of the serum MBP binding antibodies was determined in a selected set of MS and control specimens. We found that the majority of the MBP specific antibodies detected by DELFIA were of the complement-activating IgG_1 subclass (Table 2). Paired serum/CSF comparison revealed almost exclusively IgG_1 MBP antibodies in both compartments (data not shown). IgG2 anti-MBP was occasionally measured, with essentially no IgG_3 or IgG_4 anti-MBP antibodies detected.

Table 2
Binding characteristics of IgG and IgM anti-MBP antibodies in representative pediatric MS and control sera, assessed by single-dilution DELFIA, serial dilution DELFIA, and solution-phase competitive DELFIA.

Sample #	Isotype evaluated	Subject	Positive by technique	Single dilution counts	Serial dilution titer	Solution-phase competition (%)	IgG subclass of anti-MBP
59	IgG	Healthy	WED	50,286	120	n.d.	n.d.
99	IgG	Healthy	WED	39,078	80	n.d.	n.d.
172	IgG	Healthy	WED	63,888	810	n.d.	n.d.
180	IgG	Healthy	WED	63,099	300	n.d.	n.d.
196	IgG	Healthy	WED	73,345	450	n.d.	n.d.
66	IgG	Healthy	WED	97,394	240	44	IgG_1, IgG_2
101	IgG	Healthy	WED	89,632	<80	38	IgG_1
176	IgG	Healthy	WED	88,267	520	34	n.d.
69	IgG	Healthy	WED	98,228	110	5	n.d.
1	IgG	Neurological	WED	34,245	120	n.d.	n.d.
84	IgG	Neurological	WED	89,052	300	57	IgG_2
28	IgG	Other autoimmune	WED	36,030	130	n.d.	n.d.
97	IgG	Other autoimmune	WED	29,131	<80	n.d.	n.d.
50	IgG	Other autoimmune	WED	82,856	290	38	n.d.
3	IgG	MS	WED	62,721	140	n.d.	n.d.
38	IgG	MS	WED	72,900	160	n.d.	n.d.
150	IgG	MS	WED	71,628	240	n.d.	n.d.
159	IgG	MS	WED	48,686	80	n.d.	n.d.
141	IgG	MS	WED	103,000	1200	26	IgG_1
157	IgG	MS	WED	114,380	190	26	IgG_1, IgG_2
120	IgG	MS	WED	163,908	450	25	IgG_1
151	IgG	MS	WED	91,277	630	13	IgG_1
34	IgG	MS	WED	91,293	240	12	IgG_1, IgG_2
167	IgG	MS	WED	143,762	380	12	IgG_1
400*	IgG	ADEM (adult)		135,000	1800	25	IgG_1
401*	IgG	ADEM (adult)		n.d.	n.d.	35	n.d.
402*	IgG	ADEM (adult)		n.d.	n.d.	47	n.d.
500*	IgG	MS (adult)		n.d.	n.d.	9	n.d.
501*	IgG	MS (adult)		n.d.	n.d.	2	n.d.
105	IgM	Healthy	WED	50,139	120	n.d.	n.a.
191	IgM	Healthy	WED	39,653	150	n.d.	n.a.
111	IgM	Healthy	WED	50,940	200	n.d.	n.a.
55	IgM	Neurological	WED	66,466	190	35	n.a.
76	IgM	Other autoimmune	WED	44,466	160	n.d.	n.a.
67	IgM	Other autoimmune	WED	56,565	180	n.d.	n.a.
73	IgM	Other autoimmune	WED	89,064	250	20	n.a.
156	IgM	MS	WED	50,813	120	n.d.	n.a.
142	IgM	MS	WED	35,063	180	n.d.	n.a.
79	IgM	MS	WED	51,474	190	n.d.	n.a.
150	IgM	MS	WED	51,670	210	n.d.	n.a.
146	IgM	MS	WED	58,507	240	n.d.	n.a.
34	IgM	MS	WED	100,006	240	40	n.a.
143	IgM	MS	WED	60,245	290	33	n.a.
128	IgM	MS	WED	60,398	200	32	n.a.
133	IgM	MS	WED	69,276	260	17	n.a.
147	IgM	MS	WED	88,010	350	17	n.a.
164	IgM	MS	WED	113,848	750	8	n.a.
157	IgM	MS	WED	84,835	240	5	n.a.
400*	IgM	ADEM (adult)		73,853	190	28	n.a.
401*	IgM	ADEM (adult)		n.d.	n.d.	38	n.a.
402*	IgM	ADEM (adult)		n.d.	n.d.	11	n.a.
500*	IgM	MS (adult)		n.d.	n.d.	32	n.a.
501*	IgM	MS (adult)		n.d.	n.d.	12	n.a.
502*	IgM	MS (adult)		97341	300	n.d.	n.a.

*Controls—these adult-derived specimens served as assay controls. They are adult MS and ADEM serum samples known to harbor anti-MBP reactivity. W: positive by Western (immunoblot); E: positive by ELISA; D: positive by DELFIA; n.d.: not done; n.a.: not applicable.

3.5. MBP antibodies in paired CSF and serum samples

To examine the relationship between serum- and CSF-based measurements of anti-MBP antibodies, we used DELFIA to quantify these antibodies in a series of paired CSF and serum samples obtained from 25 children with a first demyelinating event (termed acute demyelinating syndrome, ADS; Supplementary Table 3). We observed a significant correlation between the anti-MBP antibody levels detected in the paired samples ($n=25$; Spearman $r=0.6049$; 95% CI 0.2756 to 0.8072, $p<0.0014$), indicating that measurements of anti-MBP antibodies in the serum of these children reasonably reflect the presence of such antibodies in their CSF, at that time.

3.6. Relationship between anti-MBP antibodies and clinical features of children with MS

We observed an interesting association between anti-MBP antibodies measured in children with MS and the clinical phenotype of their initial presentation. A total of sixteen children with MS had experienced an 'ADEM-like' first clinical episode (Supplementary Table 2). Of 22 children with MS who harbored serum anti-MBP antibodies by immunoblot (Table 1), 8 (36%) had an ADEM-like initial clinical episode, in contrast to only 8 of 69 children (12%) who did not harbor anti-MBP antibodies, representing a 3-fold increased risk (OR = 4.36, 95% CI 1.40 to 13.6; Fisher's exact $p=0.012$) of

Table 3
Affinity of serum IgG fractions binding to immature and mature forms of MBP by SPR. Saturable, dose-dependent binding responses for all purified IgG samples (0–1.2 μM) exhibited biphasic profiles over both immature and mature MBP surfaces (~400 RU amine-coupled). Representative SPR analysis in which 0.15 μM IgG sample injections were compared using a simplified bivalent analyte model (see Pol et al., 2007) to predict apparent equilibrium dissociation constants (K_D).

Category	ID	IgG sample	Immature MBP (K_D, M)	Mature MBP (K_D, M)
Control	Blank	HBS-EX	N/A	N/A
Control	Standard	Murine anti-MBP	9.93×10^{-9}	1.37×10^{-8}
Control	001-00099	Dental	1.42×10^{-7}	1.77×10^{-7}
Control	001-00101	Dental	1.38×10^{-8}	1.80×10^{-8}
Control	001-00118	Dental	3.45×10^{-8}	4.04×10^{-8}
Control	001-00125	Dental	7.44×10^{-8}	7.54×10^{-8}
Control	001-00132	Dental	4.8×10^{-8}	4.48×10^{-8}
Control	006-00010	Healthy	6.84×10^{-8}	7.50×10^{-8}
Control	001-00001	Neuro	5.33×10^{-8}	7.79×10^{-8}
Control	001-00084	Neuro	9.81×10^{-9}	1.23×10^{-8}
Control	001-00059	Ortho	4.42×10^{-8}	5.69×10^{-8}
Control	001-00066	Ortho	7.15×10^{-8}	8.42×10^{-8}
Control	001-00069	Ortho	9.91×10^{-8}	1.24×10^{-7}
Autoimmune	001-00028	Diabetes	4.37×10^{-8}	6.45×10^{-8}
Autoimmune	001-00050	JDM	8.42×10^{-9}	1.41×10^{-8}
Autoimmune	001-00097	JDM	1.45×10^{-7}	1.66×10^{-7}
MS	001-00003	MS	4.77×10^{-8}	6.45×10^{-8}
MS	001-00034	MS	1.11×10^{-7}	1.48×10^{-7}
MS	001-00038	MS	1.50×10^{-7}	1.63×10^{-7}
MS	002-00006	MS	7.01×10^{-9}	1.20×10^{-8}
MS	014-00002	MS	4.76×10^{-7}	5.14×10^{-7}
MS	014-00011	MS	4.25×10^{-8}	4.41×10^{-8}
MS	014-00012	MS	3.06×10^{-8}	3.68×10^{-8}
MS	014-00018	MS	2.48×10^{-8}	2.60×10^{-8}
MS	014-00020	MS	7.51×10^{-7}	9.83×10^{-7}
MS	017-00008	MS	1.75×10^{-7}	1.94×10^{-7}

experiencing an ADEM-like episode when anti-MBP antibodies were present. While there were no associations between the presence or absence of anti-MBP antibodies in serum of MS children and their age at first attack, disease duration, or mean relapse rate, there were significant (albeit weak) correlations between higher titers of IgG anti-MBP antibodies and both younger age at onset (Pearson's $r=-0.23$, $p=0.023$, two-tailed) and higher number of relapses (Pearson's $r=-0.21$, $p=0.046$). There was no apparent impact of concomitant use of immunomodulatory therapies on prevalence or titers of anti-MBP antibodies.

4. Discussion

Our findings first indicate that circulating anti-MBP antibodies can be present as part of the normal pediatric humoral immune repertoire in approximately 20% of children. However, these antibodies exhibit surprisingly high binding affinities to their target myelin antigen, and tend to be of the IgG1, complement fixing subclass. The presence of such high-affinity anti-myelin antibodies in the serum of children experiencing acute episodes of CNS inflammation is associated with corresponding levels of these antibodies in their CSF, and is also associated with a significantly higher likelihood of MS children presenting with more diffuse, multifocal (ADEM-like) clinical features.

An association between the presence of serum anti-myelin antibodies and more diffuse disease in children with MS does not, of course, prove 'causality', as a relatively trivial explanation could be an increased likelihood of developing anti-myelin antibodies as a consequence of more diffuse CNS insult. However, this interpretation is not supported by our present findings where, thanks to the inclusion of a large cohort of pediatric controls, we ascertained indistinguishable frequencies and binding characteristics of anti-MBP antibodies across the pediatric MS and control cohorts. We propose an alternate explanation, which reflects a "disease modifying" capacity of anti-myelin antibodies in MS: while such antibodies can be present as part of the normal humoral immune repertoire, a child who harbors them, and also happens to develop CNS inflammation, is at greater risk of experiencing a more diffuse ('ADEM-like') clinical presentation.

For this to be true, anti-MBP antibodies in these children must (i) access the CNS, and (ii) have the potential to participate in tissue injury. The first requirement is supported by our observation that in children with acute CNS inflammation for whom paired serum and CSF samples were available, levels of anti-MBP antibodies in the serum correlated well with levels of these antibodies in the CSF of the same patients. This indicates that serum levels of anti-myelin antibodies may reflect levels within the CNS, at least around the time of an active episode of CNS inflammation, when circulating antibodies could more readily access the CSF.

In relation to their potential to contribute to tissue injury, it is noteworthy that the anti-myelin antibodies measured in our pediatric cohort exhibited high binding affinities, since in other autoimmune disorders (such as myasthenia gravis and type-1 diabetes), high-affinity antibodies to self-antigens have been associated with pathogenicity (Elkon and Casali, 2008). Furthermore, these anti-myelin autoantibodies were primarily IgG_1, the subclass that can efficiently activate complement and induce antibody-dependent cell-mediated cytotoxicity.

Our results in children contrast with prior studies in adults, using the identical techniques. In our prior studies of adults with MS and controls, anti-MBP antibodies were detected only in solid phase assays but failed to appreciably bind to MBP in solution, reflecting their relatively low affinities (O'Connor et al., 2003). In contrast, using the identical techniques in the present study, we found that the anti-MBP antibodies measured in our pediatric cohorts frequently bound to MBP in both solid and soluble phase assays. The absolute K_D values we measured using the gold-standard SPR technology confirms that the anti-MBP antibodies detected in children were of high binding affinities, in a range similar to those measured for anti-pathogen antibody responses (Wrammert et al., 2008). Overall, our findings point to important differences in binding characteristics of anti-MBP antibodies between children and adults, a concept supported by the recent report of autoantibodies to MOG that were detected in a relatively high proportion of children with MS, yet not previously seen in adult MS studies (Brilot et al., 2009; McLaughlin et al., 2009).

To our knowledge, our study is also the first to investigate circulating antibodies targeting both immature and mature forms of myelin in patients with MS. Prior studies (Whitaker et al., 1992) (Klareskog et al., 2008; van Venrooij and Pruijn, 2000) have suggested that developmental defects (abnormal persistence of the immature form of MBP) may contribute as an early or perhaps even initiating target of pathogenic immune responses in MS. We first confirmed the more highly citrullinated state of immature MBP and demonstrated that it is further distinguishable from mature MBP, based on relative abundance of phosphorylated and methylated forms. However, using several complementary assays, we found that serum anti-MBP antibodies to both immature and mature forms of MBP can be detected in a substantial minority (20–25%) of all children, and that there were no differences in the frequencies, levels, or binding characteristics of either IgG or IgM anti-MBP antibodies between children with MS and control cohorts. These studies indicate that neither the immature nor mature forms of MBP are likely to represent initiating targets of the humoral response in MS. We hypothesize that the prior identification of immature MBP isoforms within adult MS lesions may reflect the expected composition of newly developing myelin, generated as part of the remyelination process.

We conclude that while MBP (in either its mature or immature form) does not appear to represent a disease-initiating CNS target of the humoral immune response in pediatric MS, high-affinity antibodies recognizing MBP can be present as part of the normal pediatric humoral immune repertoire. These anti-myelin antibodies may access

the CNS, at least in the context of active inflammation, where they could modulate the expression of disease. This may be particularly true in the youngest children, who are also known to present more commonly with ADEM-like episodes. Our findings provide a first link to classical studies in experimental autoimmune encephalomyelitis (EAE), where the mere presence of anti-myelin antibodies does not confer disease, but in the presence of such antibodies, a considerably more aggressive phenotype of CNS inflammation can be induced by the typical dose of pathogenic T cells (Lassmann et al., 1988; Schluesener et al., 1987). In the context of the human disease, our findings identify an immune mechanism that could contribute to the observed heterogeneity (Banwell et al., 2007a) in the clinical spectrum of early-onset MS and also raises the question as to whether targeting circulating antibodies, for example with plasmapheresis, may limit severity of acute attacks in a subset of children.

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Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jneuroim.2010.02.019.

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