Antibodies to native myelin oligodendrocyte glycoprotein are serologic markers of early inflammation in multiple sclerosis

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Myelin oligodendrocyte glycoprotein (MOG) is an integral membrane protein expressed in CNS oligodendrocytes and outermost myelin lamellae. Anti-MOG Abs cause myelin destruction (demyelination) in animal models of multiple sclerosis (MS); however, such pathogenic Abs have not yet been characterized in humans. Here, a method that specifically detects IgG binding to human MOG in its native, membrane-embedded conformation on MOG-transfected mammalian cells was used to evaluate the significance of these auto Abs. Compared with healthy controls, native MOG-specific IgGs were most frequently found in serum of clinically isolated syndromes ($P < 0.001$) and relapsing-remitting MS ($P < 0.01$), only marginally in secondary progressive MS ($P < 0.05$), and not at all in primary progressive MS. We demonstrate that epitopes exposed in this cell-based assay are different from those exposed on the refolded, extracellular domain of human recombinant MOG tested by solid-phase ELISA. In marmoset monkeys induced to develop MS-like CNS inflammatory demyelination, IgG reactivity against the native membrane-bound MOG is always detected before clinical onset of disease ($P < 0.0001$), unlike that against other myelin constituents. We conclude that (*i*) epitopes displayed on native, glycosylated MOG expressed *in vivo* are early targets for pathogenic Abs; (*ii*) these Abs, which are not detected in solid-phase assays, might be the ones to play a pathogenic role in early MS with predominant inflammatory activity; and (*iii*) the cell-based assay provides a practical serologic marker for early detection of CNS autoimmune demyelination including its preclinical stage at least in the primate MS model.

allergic encephalomyelitis | autoimmunity | clinically isolated syndrome | demyelination

Multiple sclerosis (MS) is an immune-mediated, chronic demyelinating disorder of CNS white matter mediated by $CD4^+$ myelin-reactive T helper 1 cells (1). Humoral immunity may play a role in MS pathogenesis, as suggested by intrathecal Ab synthesis and oligoclonal B cell expansion, observations of Ab deposition associated with myelin vacuolization in MS plaques (2), and involvement of IgG and complement deposition (3). Myelin oligodendrocyte glycoprotein (MOG) is a target myelin antigen for both humoral and cellular CNS-directed immune responses. The full-length protein contains 218 aa and two predicted transmembrane domains, is posttranscriptionally processed as suggested by apparent electrophoretic mobility, and is a potential site for glycosylation, phosphorylation, isoprenylation, and myristoylation (4). The encephalitogenic properties of MOG are believed to result from the extracellular location of its IgV-like domain on the outermost myelin lamellae, which makes it an exposed target accessible to initial autoimmune attack on compact myelinated axons (5). In both rodent and primate MS models of experimental allergic encephalomyelitis (EAE), Abs against MOG directly induce demyelination (6, 7). Moreover, certain Abs against MOG have been proposed as predictors of early conversion to clinically definite MS in the context of a first demyelinating event (8).

A common problem encountered in human studies of humoral immunity against MOG is that accurate detection of Ab depends highly on the conformation of the antigens used for detection. Previous studies, either in MS or EAE, have not fully characterized the structural features of the proteins used as Ab targets *in vitro* and have reported conflicting findings, in part because of the different folded forms of this poorly water-soluble antigen. The existing assays detecting anti-MOG Abs use various MOG preparations, recombinant, native-purified, or *in vitro*-translated proteins of variable length including short peptides (4, 8–11). None of these methods takes into account the specific tertiary structure of the folded MOG as it is presented to the immune system *in vivo*, e.g., in association with a hydrophobic, lipid-rich bilayer membrane environment. Therefore, these techniques may fail to detect reactivity against epitopes displayed by native MOG expressed *in situ* on myelin sheaths, as it would be upon an initial immune response.

Here, we describe a cell-based assay that specifically measures Abs directed against conformationally folded, cell membrane-expressed human MOG (designated $hMOG_{cme}$), and evaluate the relative incidence of these antinative MOG Abs in serum of humans and *Callithrix jacchus* marmosets with MS-like EAE. We find that $hMOG_{cme}$ Abs predominate during clinically isolated syndrome (CIS) and relapsing-remitting MS (RRMS) and can be detected during the preclinical stage of EAE. These findings underscore the potential value of antinative MOG Abs as a practical candidate biomarker for detecting MS at its early, inflammatory stage.

Results

Chinese Hamster Ovary (CHO)-MOG Assay (MOG_{cme}) Validation. Fig. 1 shows high levels of MOG_{cme} expression, as demonstrated by staining of MOG-transfected CHO cells with the monoclonal anti-MOG Ab 8-18C5. Detection of $hMOG_{cme}$-specific Abs with this cell-based assay was sensitive because a concentration of <1 ng/ml of 8-18C5 produced a binding ratio (BR) >1.5 (data not shown). Staining with a positive control serum (patient 1158) is shown in Fig. 1*C*. This control was used in each assay to

Conflict of interest statement: P.H.L., T.M., H.-C.v.B., and C.P.G. are coinventors of an international patent application that contains claims to methods of diagnostics and prognosis of multiple sclerosis. Such methods may be ultimately commercialized.

Abbreviations: MOG, myelin oligodendrocyte glycoprotein; $rMOG_{125}$, recombinant rat $MOG_{aa1-125}$; $hMOG_{125}$, recombinant human $MOG_{aa1-125}$; $hMOG_{cme}$, cell-membrane expressed human MOG; CHO, Chinese hamster ovary; ntCHO, nontransfected CHO; EAE, experimental allergic encephalomyelitis; CIS, clinically isolated syndrome; MS, multiple sclerosis; RRMS, relapsing-remitting MS; SPMS, secondary progressive MS; PPMS, primary progressive MS; HC, healthy control; BR, binding ratio; Gmean, geometric mean intensity.

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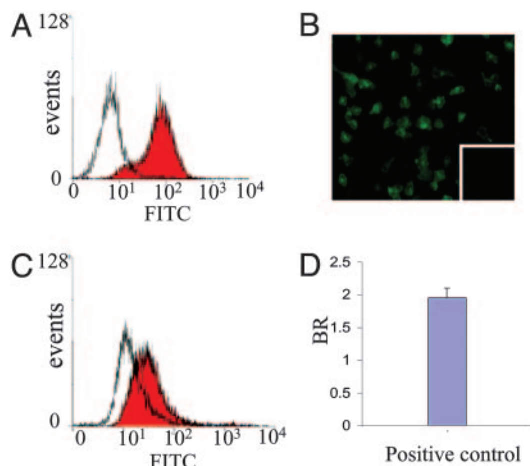

Fig. 1. Cell-based (hMOG$_{cme}$) assay. (*A* and *B*) Staining of MOG-transfected CHO cells with anti-MOG Ab 8-18C5 (0.5 μg/ml) and detection by FACS (*A*) and immunofluorescence (*B*). (*B Inset*) Negative control omitting primary Ab. (*C*) Positive control patient serum (RRMS 1158, 1:10) displaying a clear shift for MOG-transfected CHO cells (filled trace) when compared with ntCHO cells (open trace). (*D*) Mean BR (BR $\pm$ SEM) calculated as the Gmean from nine independent assays.

normalize for interassay variability and minimize experimental errors such as variation in surface expression of MOG. The mean ($\pm$ SEM) BR to hMOG$_{cme}$ of this control from nine independent experiments was 1.96 $\pm$ 0.145 (Fig. 1*D*), and the interassay coefficient of variation was 22%. The intraassay coefficient of variation (quadruplicate) was 3.2%. In each assay analyzing human serum, the binding against nontransfected CHO (ntCHO) cells was used as background control.

Characterization of Exposed Epitopes of hMOG$_{cme}$. We analyzed the binding properties of monoclonal, recombinant marmoset Fab Ab fragments produced against the nonglycosylated extracellular domain of recombinant rat MOG$_{aa1–125}$ (rMOG$_{125}$). Four Fabs, designated M3-8, M26, M3-24, and M3-31, were selected by their ability to recognize rMOG$_{125}$ in ELISA, and because they recognize distinct conformationally defined epitopes (12). The M3-31 and M26 Fabs strongly stained the MOG-transfected CHO cells identical to 8-18C5 (0.5 μg/ml) (Fig. 2 *A* and *B*). On the contrary, no binding was observed for the two other Fabs, M3-24 and M3-8 (Fig. 2 *C* and *D*). These results indicate that very specific epitopes of MOG are expressed on the MOG-transfected CHO cells and do not overlap with the other epitopes displayed by rMOG$_{125}$ on solid ELISA support. The observation that only three of five monoclonal reagents tested bind to the transfected cells also renders a nonspecific binding effect unlikely.

IgG Reactivity in Human Serum. Compared with age-matched healthy controls (HCs), the titers of IgG directed against membrane-bound hMOG$_{cme}$ were most significantly increased in CIS ($P < 0.001$). Increased titers were also present in RRMS and secondary progressive MS (SPMS) subtypes, compared with HC ($P < 0.01$ and $P < 0.05$, respectively) (Fig. 3). The differences were also significant when comparing primary progressive MS (PPMS) with all other subtypes [$P < 0.001$ (CIS), $P < 0.01$ (RRMS), and $P < 0.05$ (SPMS)]. No statistical difference was found between PPMS and HC (*P* not significant) or between the CIS, RRMS, or SPMS subtypes when paired comparisons were made. No treatment-related difference was found.

IgG Reactivity in Marmoset EAE. Eleven *C. jacchus* marmosets immunized with human white matter were tested for plasma

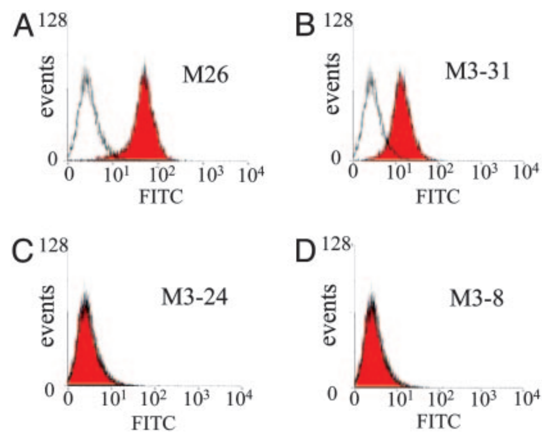

Fig. 2. Binding characteristics of MOG-specific marmoset recombinant Fab fragments. Binding of monoclonal Fabs specific for rMOG$_{125}$ (0.5 μg/ml) by FACS against hMOG$_{cme}$. Background is represented by the binding to the ntCHO cells (open traces) and compared with the binding to MOG-transfected CHO cells (filled traces). Both Fabs M26 (*A*) and M3-31 (*B*) strongly recognize an epitope displayed on hMOG$_{cme}$, in contrast to Fabs M3-24 (*C*) and M3-8 (*D*).

reactivity against hMOG$_{cme}$ on MOG-transfected CHO cells. For these serial studies, the first time point of Ab detection (i.e., serum conversion) was compared with the appearance of the first clinical signs of EAE. Three animals (U30-00, UO61-02, and UO53-01) were killed before onset of neurological deficits (preclinical disease), but exhibited CNS inflammation and blood-brain-barrier breakdown as demonstrated by cerebrospinal fluid pleocytosis (mean cerebro-spinal fluid mononuclear cells = 173 per μl; range 80–340 per μl). Serum reactivity against hMOG$_{cme}$ was consistently detected in the earliest blood sample obtained after immunization (mean $\pm$ SD = 14 $\pm$ 2 days; range = 13–18 days) (Table 1) and was clearly present in each animal before the appearance of any clinical sign (mean $\pm$ SD = 21 $\pm$ 9 days; range = 16–43 days). The difference between time of appearance of serum IgG reactivity to hMOG$_{cme}$ and appear-

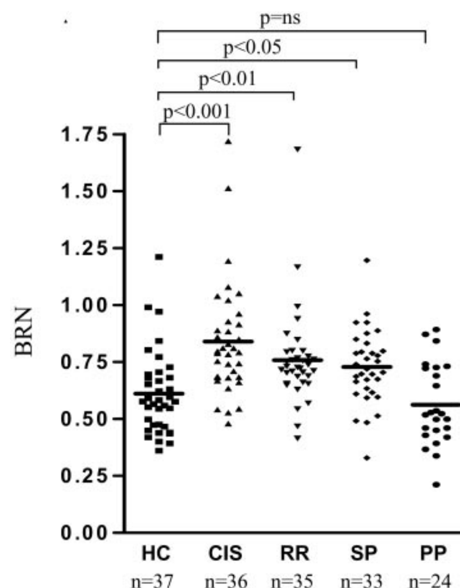

Fig. 3. Analysis of human serum IgG reactivity against hMOG$_{cme}$. BR normalized (BRN) is the Gmean value of IgG binding to MOG-transfected CHO cells divided by that of IgG binding to ntCHO cells, normalized to the value of the positive control tested in each plate. Horizontal bars = median. See text for details.

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Table 1. Time course of *C. jacchus* marmoset EAE and serum IgG binding against $hMOG_{cme}$

Animal no.	Clinical onset dpi	Death dpi	Maximal clinical score (dpi)	α-$hMOG_{cme}$ Ab dpi
UO62–02	43	97	3 (84)	18
UO50–01	21	86	2 (57)	14
UO50–00	21	78	3 (38)	14
UO52–01	21	52	3 (40)	18
UO25–00	21	61	2 (57)	14
UO57–02	32	82	1.5 (73)	18
UO21–99	18	23	1.5 (23)	13
UO23–00	16	31	2 (28)	15
UO30–00	Preclinical	23	0	13
UO61–02	Preclinical	31	0	14
UO53–01	Preclinical	38	0	16
Median ± SD	21 ± 9	52 ± 28	2 ± 1 (49 ± 22)	14 ± 2

dpi, day postimmunization.

ance of clinical signs was highly significant ($P < 0.0001$) (Fig. 4). Reactivity was not detected in preimmune plasma.

Comparison of Human IgG Binding on $hMOG_{125}$ and $hMOG_{cme}$. The serum binding characteristics of the CIS cohort ($n = 36$) were tested by ELISA using recombinant human $MOG_{aa1-125}$ ($hMOG_{125}$) and compared with $hMOG_{cme}$ reactivity by FACS on the MOG-transfected CHO cells. Although some CIS patients displayed high reactivity against $hMOG_{125}$, unlike for $hMOG_{cme}$ reactivity, there was no statistical significant difference between CIS and HCs (data not shown). By linear regression and comparison test, results from these two assays showed no correlation (*P* not significant), indeed suggesting that different epitopes are detected by both assays (Fig. 5*A*).

Specificity of MOG and Differential MOG-Epitope Binding in Human Serum. To discriminate the epitopes displayed by $hMOG_{125}$ from those displayed on $hMOG_{cme}$ on MOG-transfected CHO cells, we performed a series of preabsorption experiments with two sera, both representative of early and inflammatory forms of MS: the positive control used in our cell-based assay (RRMS 1158) and a CIS patient displaying a high reactivity to both

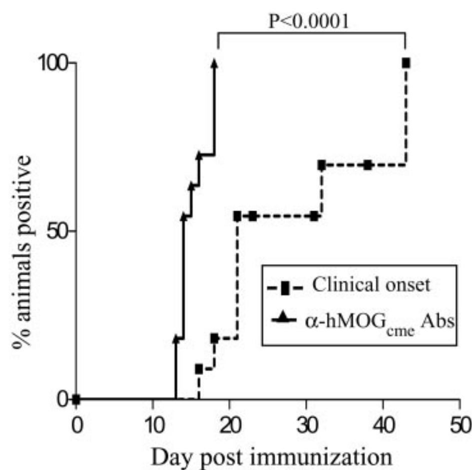

Fig. 4. Time course of serum IgG directed against $hMOG_{cme}$ in marmoset EAE. Results are from 11 EAE *C. jacchus* marmosets immunized with human white matter, 3 of which were killed before onset of clinical disease. First occurrence of serum IgG directed against $hMOG_{cme}$ is compared with time of clinical onset of EAE in a Kaplan–Meier survival plot. Please see text and Table 1 for details.

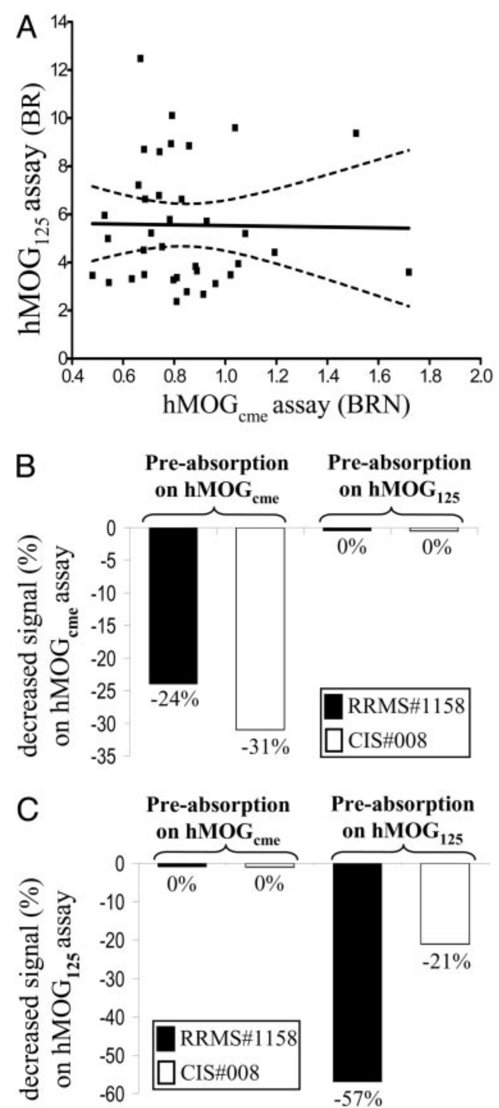

Fig. 5. Selective epitope presentation on $hMOG_{cme}$. (*A*) Binding to $hMOG_{125}$ by ELISA compared with binding to $hMOG_{cme}$ by FACS in the CIS cohort ($n = 36$). By linear regression analysis, there is no correlation between the results of these two methods (*P* not significant, $r^2 = 0.00023$, Spearman *r*; straight line is the linear regression curve; dotted line indicates 95% confidence interval) even when clear serum reactivity is present in both assays. BRN, BR normalized. (*B* and *C*) Preabsorption of serum on either $hMOG_{cme}$ (*Left*) or $hMOG_{125}$ (*Right*), followed by testing by FACS (*B*, $hMOG_{cme}$) or ELISA (*C*, $hMOG_{125}$). Preabsorption on $hMOG_{125}$ or $hMOG_{cme}$ only altered the reactivity in the corresponding system of detection. See *Materials and Methods* for additional details.

$hMOG_{cme}$ and $hMOG_{125}$ (CIS 008). Preabsorption against ntCHO cells served as control in the $hMOG_{cme}$ assay (FACS of MOG-transfected CHO cells), and preabsorption against 1% BSA served as a control for the $hMOG_{125}$ ELISA assay. Compared with these controls, preabsorption of either sample on $hMOG_{125}$ did not affect $hMOG_{cme}$ reactivity. Preabsorption on $hMOG_{cme}$ resulted in a decrease in BR (31% for CIS 008 and 24% for RRMS 1158), when tested with $hMOG_{cme}$ expressed on the MOG-transfected cells (Fig. 5*B*). Similarly, when tested on $hMOG_{125}$, no change in reactivity occurred when samples were preabsorbed on the MOG-transfected cells ($hMOG_{cme}$). On the contrary, the samples preabsorbed on $hMOG_{125}$ displayed a decrease in BR, by 21% and 57%, respectively, when tested on $hMOG_{125}$ (Fig. 5*C*). These experiments unequivocally demon-

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strate that $hMOG_{cme}$ and $hMOG_{125}$ display separate epitopes of the MOG protein.

Discussion

Methods to study Ab reactivity to MOG (ELISA, Western blot, liquid-phase assay) commonly use linear peptides, polypeptide, or partially refolded glycosylated MOG. The exact conformation of MOG displayed in these assay systems is difficult to assess and control and may result in the display of some, or partially, aberrant MOG epitopes that are not exposed under physiological conditions *in vivo*. The disease relevance of these Abs is therefore uncertain, as apparent from somewhat conflicting results in previous reports (2, 8–11, 13–16). We show here that Abs against native glycosylated MOG expressed on mammalian cells are commonly detected in MS serum with high sensitivity (<1 ng). Specificity is established by the selective binding of three of five monoclonal anti-MOG Abs tested in this system (murine 8-18C5 and marmoset Fabs M3-31 and M26). Thus, as is the case for these mAbs (9, 12, 13) the $hMOG_{cme}$ assay measures Abs that bind to conformational epitopes of MOG.

Our results show that there is no correlation in the CIS cohort between serum reactivity against $hMOG_{125}$ (solid-phase ELISA) and $hMOG_{cme}$ (MOG-transfected CHO cells), and preabsorption demonstrates that there is no cross-reactivity between epitopes of MOG displayed in these two different assays (Fig. 5). This finding also argues, along with the significant signal quenching in the cell-based assay achieved only by preabsorption on $hMOG_{cme}$ (MOG-transfected cells), and not by preabsorption on either $hMOG_{125}$ or ntCHO cells, against a nonspecific "sticky" effect of MS serum. The $hMOG_{cme}$ assay is unique because it allows the testing of IgG reactivity directed against epitopes presented by the native glycosylated and conformational structure of MOG as it is expressed on intact myelin sheath or oligodendrocytes and subject to membrane lipid–protein interactions, which have been shown to be critical for maintenance of myelin structure and epitope exposure (17).

Analysis of reactivity against $hMOG_{cme}$ on the MOG-transfected CHO cells in the different MS clinical subtypes showed a very prominent response in CIS, RRMS, and to a lesser degree SPMS, compared with HC and PPMS. There is therefore a humoral immune response specifically directed against intact MOG expressed on myelin oligodendrocytes in those groups of patients. The predominance of $hMOG_{cme}$-specific Abs in CIS suggests that these Abs represent early stages of the immune response against intact (as opposed to degraded) myelin, and thus may represent a marker of inflammatory phases of disease related to blood-brain-barrier opening and/or molecular mimicry. Our results are in partial agreement with a recent report showing an increase of Ig response directed against *ex vivo* glycosylated-native-MOG in first demyelinating events (14). RRMS is the most common MS subtype that includes ≈85% of the patients at initial presentation. The secondary progressive pattern is known to follow RRMS in ≈50% of the cases after 10 years of disease activity (18, 19). Thus, these two subtypes might be considered as a continuous process starting with a common pathophysiological origin. Although we did not include a group of other neurological disorders in the current study, we have compared MS clinical subtypes among themselves and clearly demonstrated that the $hMOG_{cme}$-specific Ab response is restricted to early forms. It is of importance to note that the PPMS cohort does not show elevated serum IgG against $hMOG_{cme}$ and that significant differences in antibody status also exist between SPMS and PPMS, which implies that the serum level of these Abs or lack thereof is not solely related to a progressive course of disease. The HC subjects were age-matched with the CIS group, indicative that the heightened response in CIS is disease-specific and not related to a younger age in this cohort. The lack of heightened Ab responses against $hMOG_{cme}$ in PPMS is in contrast with the increased IgG reactivity against recombinant $rMOG_{125}$ and against neurons in this disease subtype (unpublished data and ref. 20). Abs against galactocerebroside, the major myelin glycolipid, are not found in PPMS but are associated mostly with established relapsing-remitting and secondary-progressive forms (21). It is thus becoming increasingly apparent that Ab responses against myelin antigens may follow patterns that reflect a combination of underlying cause, antigen exposure, and secondary immune responses. These patterns of humoral reactivity, rather than a classification based on clinical criteria (RRMS, SPMS, or PPMS) can be exploited to refine our understanding of disease stage, cause, and prognosis.

The high prevalence of $hMOG_{cme}$-reactive Abs in CIS, i.e., contemporary of the first clinically apparent event for MS, is in sharp contrast to other antimyelin Abs, such as those directed against glycolipids that predominate in established MS (21). This observation has two important implications: first, it suggests that $hMOG_{cme}$-reactive Abs may be implicated in the early pathogenesis of disease. Engagement of membrane-embedded MOG by the mAb 8-18C5, which as shown in the current study binds $hMOG_{cme}$ with a high affinity, has been shown to induce MOG phosphorylation in oligodendrocytes, leading to pronounced morphological changes with potentially demyelinating effects (22). Second, and/or alternatively, our findings also suggest that $hMOG_{cme}$-reactive Abs may be useful to help diagnose MS at its earliest stages. To consolidate this contention, the $hMOG_{cme}$ assay was used to study the time course of the Ab response against $hMOG_{cme}$ in marmoset EAE induced by immunization with human white matter. In these animals, serum reactivity against $hMOG_{cme}$ was always detected before clinical onset, contrary to anti-myelin basic protein and antigalactocerebroside Abs that occur at later stages (21). Because the immunizing antigen contained native MOG similar in conformation to $hMOG_{cme}$, these findings imply that the $hMOG_{cme}$-reactive Abs are the ones that initiate and/or first result from active demyelination. Regardless of whether they are causative or not, $hMOG_{cme}$-reactive Abs clearly represent a valuable biomarker for disease activity and, at least in the MS model, subclinical disease.

It is of great interest to note that the marmoset Fabs M3-31 and M26, which were obtained from an animal with overt clinical signs of EAE immunized with $rMOG_{125}$ and had an established anti-MOG Ab response, are the only ones among those tested that recognize $hMOG_{cme}$. In our previous studies of human MS, using a highly specific competition assay between human serum IgG and marmoset Fabs we found that Fabs M3-24 and M3-8 can compete with serum IgG from patients with established MS, but we have so far not been able to demonstrate any competition between human IgGs and either M3-31 or M26 (12). Although further studies are needed to examine whether IgG purified from patients with a CIS does compete with Fabs M3-31 and M26, these data suggest that the epitopes defined by these two Fabs are the ones targeted by early humoral responses in MS, whereas the ones defined by M3-8 and M3-24 may be part of the Ab response at a later stage.

Taken together, our results strongly suggest that analogous to certain serologic markers that are predictive of type I diabetes (23) anti-$hMOG_{cme}$ Abs could be used in humans as a biomarker to diagnose MS or MS risk. Further studies are needed to validate this biomarker and understand the benefits and information that in combination with other Ab profiling techniques (8, 21) it could provide to scientists, treating neurologists, and individuals with suspected or established MS.

Materials and Methods

Patients. Ninety-two patients with clinically definite MS (Poser criteria) (24), 36 patients with CIS, and 37 HCs were recruited from the University of California, San Francisco MS Center, and

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Table 2. Clinical characteristics of patients and HCs

Patients	No. of cases	Female/male ratio	Median age, yr (range)	Median disease duration, yr (range)	Median EDSS (range)
CIS	36	24/12	32 (18–49)	0.3 (0.1–3)	2 (0–4)
RRMS	35	21/14	41 (19–61)	2.4 (0.6–22.2)	2.5 (0–6.5)
SPMS	33	19/14	46 (36–60)	11 (2–32)	6 (2.5–8.5)
PPMS	24	16/08	54 (40–65)	6.3 (0.8–18.8)	4.5 (2.5–8)
HC	37	24/13	35 (21–63)	—	—

EDSS, expanded disability status scale (25).

the MS Center of Pamplona, Spain (CIS). All investigations were conducted according to the Declaration of Helsinki. Blood was obtained by venipuncture after informed consent in full compliance with the Institutional Review Board, and clotted serum was stored at −40°C until use. Patients were classified as RRMS ($n = 35$), SPMS ($n = 33$), and PPMS ($n = 24$) MS by clinical history (24). A CIS was defined by a first clinical event indicative of demyelination with no history of previous neurological symptom. Age, gender, disease duration, and disability state [Expanded Disability Status Score (EDSS) (25)] were recorded at time of sampling. HCs were chosen to match sex and age of the CIS group. The median age, disease duration, and EDSS were higher for the SPMS group than for the RRMS and CIS groups (Table 2). All RRMS and 19/33 SPMS patients were treated with IFN-β. Two of the PPMS patients were treated with mitoxantrone and monthly pulsed steroids.

Animals. *C. jacchus* marmosets were cared for in accordance with the guidelines of the Institutional Animal Care and Usage Committee. EAE was induced by immunization with 100 mg of human white matter, which contains native, membrane-embedded MOG homogenized in complete Freund's adjuvant as described (26). Plasma was obtained from EDTA-anticoagulated blood at baseline and 2- to 4-week intervals and stored at −40°C. The animals were scored every other day for the development of clinical signs by using a published scale (27).

Preparation of MOG-Transfected Cells. CHO cells were transfected with a full-length construct corresponding to the major α-1 form of human MOG as described (28). CHO cells were cultured in T-225 flasks (Costar), in RPMI medium 1640 supplemented with 10% FCS, 1× Glutamax, 1 mM sodium pyruvate, and 50 μg/ml gentamycine. G418 (500 μg/ml, GIBCO) was added to the medium of transfected cells. Stable surface expression of MOG was verified by immunofluorescence and FACS on transfected cells after multiple passes and washes; cells were used for flow cytometry (FACS) assay when a confluence of 80–90% was reached. To check for surface expression of $hMOG_{cme}$, 3×10^4 MOG-transfected CHO cells were deposited on a slide and fixed with 100% methanol for 5 min at −20°C. After blocking with PBS containing 2% BSA and 2% FCS for 30 min, cells were incubated 1 h at 37°C with the mouse monoclonal anti-MOG Ab 8-18C5 (5 μg/ml, gift of C. Linington, University of Aberdeen, Aberdeen, Scotland). Fluorescence was revealed after 1-h incubation at 37°C by a goat anti-mouse IgG FITC Ab (Sigma) and examined under a fluorescence microscope (Nikon Eclipse E600). Negative controls were done with secondary Ab alone.

Serum IgG Reactivity. Cells were trypsinized, diluted in FACS buffer (PBS, 0.1% Na azide, and 2% FCS), and plated in a 96-well plate (Costar) at a density of 200,000 per well. After blocking in FACS buffer containing 10% FCS for 15 min at 4°C, cells were washed and human serum (1:10) was added for 1-h incubation at 4°C. After washing, cells were incubated with a goat anti-human IgG FITC (Caltag, South San Francisco, CA) at the recommended concentration for 30 min at 4°C. After a final wash, cells were resuspended in FACS buffer containing propidium iodide (Molecular Probes) at 2 μg/ml and gently shaken. Samples were kept on ice and analyzed by gating the selected live cell population (10^4 cells) within 1 h of harvesting by trypsinization. Each FACS experiment included an internal positive control consisting of the monoclonal anti-MOG 8-18C5 Ab (0.5 μg/ml) and rabbit anti-mouse FITC (DAKO) as secondary Ab. For each sample, the geometric mean intensity (Gmean) of FITC (WINMDI 2.8 software) was measured for MOG-transfected CHO cells and compared with that of ntCHO cells. The BR was calculated as the Gmean for MOG-transfected CHO cells divided by the Gmean for ntCHO. To compare different assays, for each sample the BR was normalized to that of a human positive control (RRMS 1158) included in each experiment.

For studies in marmosets, MOG-transfected CHO cells were incubated for 1 h at 4°C with marmoset serum diluted 1:100. FITC-conjugated Ab against whole monkey IgG (Sigma) diluted at 1:100 was used as secondary Ab and incubated 30 min at 4°C. FACS analysis was performed as described above. IgG binding against $hMOG_{cme}$ was considered positive when the BR (Gmean preimmunization/Gmean time point tested) was >1.5.

Differential Reactivity of Monoclonal Fab Fragments. Recombinant Fabs were derived from a *C. jacchus* marmoset immunized with $rMOG_{1–125}$ produced in *Escherichia coli* ($rMOG_{125}$) (12). Fabs were diluted in FACS buffer at 0.5 μg/ml and added to ntCHO or MOG-transfected CHO cells. FITC-conjugated Ab against whole monkey IgG (Sigma) diluted at 1:100 was used as secondary Ab for 30 min at 4°C. FACS was performed as described above.

ELISA Assay. $hMOG_{125}$ expressed in *E. coli* was coated overnight on polystyrene microtiter plates at 0.5 μg per well (Maxisorb, Nunc). After washing and blocking with 1% BSA in PBS + 0.05% Tween (BSA-PBS-T) for 2 h at room temperature, sera (1:200) were diluted in BSA-PBS-T and added to the plate. Ab binding was detected by an alkaline phosphatase AP-labeled goat-anti-human IgG (Sigma) for 1 h at room temperature. Plates were developed with *para*-nitrophenyl phosphate (Moss, Pasadena, MD) for 30 min in the dark at room temperature and read at 405 nm in a microplate reader (SpectraMax, Molecular Devices). Results were expressed as BR, i.e., signal over BSA background.

Preabsorption of Sera on $hMOG_{cme}$ and $hMOG_{125}$. To further validate the cell-based assay and eliminate the possibility of nonspecific binding effects from either MS serum or transfection procedures, we conducted binding experiments after preincubation of serum with the respective antigens. For $hMOG_{cme}$ preabsorption, 5×10^6 MOG-transfected CHO cells and ntCHO cells were separately incubated with serum diluted 1:10 for 1 h at room temperature with gentle agitation. After centrifugation at 900 × *g* for 2 min, supernatant were collected and preabsorption was

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repeated three times in total with fresh cells. After the final preabsorption, supernatants were centrifuged at 3,600 $\times g$ for 5 min and collected for subsequent experiments.

For $hMOG_{125}$ preabsorption, ELISA plates were coated with either 1 μg BSA or 0.5 μg $hMOG_{125}$ overnight and blocked in 1% BSA in PBS plus 0.05% Tween for 2 h, then sera were incubated 1 h. Supernatants were collected and preabsorption was repeated eight times in total with fresh $hMOG_{125}$. After the final preabsorption step, supernatants were collected as above.

Data Analysis. Statistical analyses were performed by using Kruskal–Wallis with Dunn's post hoc test for multiple comparisons for interpatient group differences. Interassay correlation was analyzed with Spearman *r* correlation. Survival analysis for time-dependent variables was assessed by Kaplan–Meyer analysis and the Cox proportional hazard model (PRISM 3.0).

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