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

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Communicated by Jacob N. Israelachvili, University of California, Santa Barbara, CA, December 15, 2005 (received for review July 6, 2005)

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 (specific IgGs were most frequently found in serum of clinically isolated syndromes (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 (MOGcme) Validation. Fig. 1 shows high levels of MOGcme expression, as demonstrated by staining of MOG-transfected CHO cells with the monoclonal anti-MOG Ab 8-18C5. Detection of MOGcme-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. 1C. 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; MOGcme, recombinant rat MOGcme; hMOGcme, recombinant human MOGcme; hMOGcme, cell membrane-expressed human MOG; CHO, Chinese hamster ovary; mCHO, 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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normalize for interassay variability and minimize experimental errors such as a variation in surface expression of MOG. The mean (± SEM) BR to hMOGcme of this control from nine independent experiments was 1.96 ± 0.145 (Fig. 1D), 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 hMOGcme. We analyzed the binding properties of monoclonal, recombinant marmoset Fab Ab fragments produced against the nonglycosylated extracellular domain of recombinant rat MOGaa1-125 (rMOG125). Four Fabs, designated M3-8, M26, M3-24, and M3-31, were selected by their ability to recognize rMOG125 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. 2A 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 rMOG125 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 hMOGcme 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 reactivity against hMOGcme 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 cerebrospinal fluid mononuclear cells = 173 per μl; range 80–340 per μl). Serum reactivity against hMOGcme was consistently detected in the earliest blood sample obtained after immunization (mean ± SD = 14 ± 2 days; range = 13–18 days) (Table 1) and was clearly present in each animal before the appearance of any clinical sign (mean ± SD = 21 ± 9 days; range = 16–43 days). The difference between time of appearance of serum IgG reactivity to hMOGcme and appear-

Fig. 1. Cell-based (hMOGcme) assay. (A and B) Staining of MOG-transfected CHO cells with anti-MOG Ab B-18CS (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 115B, 1:10) displaying a clear shift for MOG-transfected CHO cells (filled trace) when compared with ntCHO cells (open trace). (D) Mean BR (BR ± SEM) calculated as the Gmean from nine independent assays.

Fig. 2. Binding characteristics of MOG-specific marmoset recombinant Fab fragments. Binding of monoclonal Fabs specific for rMOG125 (0.5 μg/ml) by FACS against hMOGcme. 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 hMOGcme, in contrast to Fabs M3-24 (C) and M3-8 (D).

Fig. 3. Analysis of human serum IgG reactivity against hMOGcme. 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.
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} \((\text{hMOG}_{125})\) 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 \((P \text{ not significant})\), indeed suggesting that different epitopes are detected by both assays (Fig. 5A).

**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 \((\text{RRMS} 1158)\) and a CIS patient displaying a high reactivity to both hMOG_{cme} and hMOG_{125} \((\text{CIS} \#008)\). Preabsorption against ntCHO cells served as control in the hMOG_{cme} assay \((\text{FACS of MOG-transfected CHO cells})\), and preabsorption against 1% BSA served as a control for the hMOG_{125} ELISA assay \((P \text{ not significant})\). Preabsorption on hMOG_{125} or hMOG_{cme} only altered the reactivity in the corresponding system of detection. See Materials and Methods for additional 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 \text{ not significant}, r^2 = 0.00023, \text{Spearman } r); \text{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. \((B \text{ and } C)\) Preabsorption of serum on either hMOG_{cme} \((\text{Left})\) or hMOG_{125} \((\text{Right})\), followed by testing by FACS \((B, \text{hMOG}_{cme})\) or ELISA \((C, \text{hMOG}_{125})\). Preabsorption on hMOG_{125} or hMOG_{cme} only altered the reactivity in the corresponding system of detection. See Materials and Methods for additional details.
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 CHO 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 IgG 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 antmyelin 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 antialgalactocerebroside 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...
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 symptoms. 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. The median 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 higher for the SPMS group than for the RRMS and CIS groups. 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 higher for the SPMS group than for the RRMS and CIS groups.

Animals. C. jaccus marmosets were cared for in accordance with the guidelines of the Institutional Animal Care and Usage Committee. EAE was induced by immunization with 100 µg 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, 1000 µM glutamine, 1 mM sodium pyruvate, and 50 µg/ml gentamycin. 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 passages and washes; cells were used for flow cytometry (FACS) assay when a confluence of 80–90% was reached. To check for surface expression of hMOGcme, 3 × 10^6 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 8-18CS (5 µg/ml, gift of C. Lintong, 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 (10^6 cells) within 1 h of harvesting by trypsinization. Each FACS experiment included an internal positive control consisting of the monoclonal anti-MOG 8-18CS Abs (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 hMOGcme 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. jaccus marmoset immunized with rMOG1125 produced in Escherichia coli (rMOG1125) (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. hMOG125 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 hMOGcme and hMOG125. To further validate the cell-based assay and eliminate the possibility of nonspecific binding effects from either MS serum or transfusion procedures, we conducted binding experiments after preincubation of serum with the respective antigens. For hMOGcme 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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<th>Patients</th>
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<td>35 (21–63)</td>
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EDSS, expanded disability status scale (25).

Table 2. Clinical characteristics of patients and HCs
repeated three times in total with fresh cells. After the final preabsorption, supernatants were centrifuged at 3,600 × g for 5 min and collected for subsequent experiments.

For hMOG125 preabsorption, ELISA plates were coated with either 1 μg BSA or 0.5 μg hMOG125 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 hMOG125. 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).

We thank Paul Dazin, Ishita Barman, and Salomon Martinez for expert technical help and neurologists and staff at the University of California San Francisco MS center for sample collection. This work was supported by National Multiple Sclerosis Society Grants FG-1562-A-1 (to P.H.L.), FG1476-A-1 (to T.M.), and RG3435-A-7 (to C.P.G.); Swiss National Foundation Grant PBGB2-109218 (to P.H.L.); National Institutes of Health Grants 5R01NS046678 and 5R01AI043073; the Cure MS Now Foundation; and the Lunardi Foundation.