The glycopeptide CSF114(Glc) detects serum antibodies in multiple sclerosis

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Abstract

Synthetic glycopeptides have the potential to detect antibodies in multiple sclerosis (MS). In the present study, we analyzed the antibodies (IgM class, IgG class and IgG subclasses) to the synthetic glycopeptide CSF114(Glc) in the serum of 186 MS patients, 166 blood donors (BDs), 25 patients affected by meningitis/encephalitis, 41 affected by systemic lupus erythematosus (SLE) and 49 affected by rheumatoid arthritis (RA). The IgM antibody level to CSF114(Glc) was significantly increased in MS patients versus BDs ($p < 0.001$) or versus other autoimmune diseases (SLE or RA, $p < 0.001$). The IgG response was restricted to the subclass IgG2. IgM antibodies to CSF114(Glc) were found in 30% of relapsing/remitting MS patients and, at lower levels, in subjects affected by meningitis/encephalitis. The study of antibodies to CSF114(Glc) is a new, potential immunological marker of MS.
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1. Introduction

Multiple sclerosis (MS) is an autoimmune disease with an unpredictable course and a difficult prognostic evalua-tion. The pathology of MS is believed to be heterogeneous and different effector immune mechanisms may lead to the formation of inflammation and demyelination within the central nervous system (CNS) lesions (Kornek and Lassmann, 2003; Lassmann et al., 2001). Magnetic resonance imaging (MRI) has proven to be a sensitive technique in detecting MS lesions and it is the only accepted marker of the disease (Fazekas et al., 1999). Nonetheless, understanding the relationship between the

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detection of MRI changes and brain pathology is at present incomplete (Wayne Moore, 2003). Therefore, it is essential to identify new markers able to help in management of the disease. Not surprisingly, just a few immune tests have been linked to a specific pathogenetic step of the disease (Bielekova and Martin, 2004).

In the past decade, a large body of evidence has highlighted the important role of autoreactive B cells and autoantibodies in the course of MS, at least in relapsing/ remitting (RR) patients (Cross et al., 2001). Interesting results were obtained studying the autoantibody response in experimental allergic encephalomyelitis (EAE), whereas antibodies to myelin glycoproteins can induce significant and prominent demyelination and severe disease (Glynn and Linington, 1989; Linington et al., 1988). Previous findings also support this pathogenetic mechanism in humans. First, oligoclonal IgG are synthesized in brain lesions and diffuse into cerebrospinal fluid (CSF), an alteration considered of diagnostic and prognostic value in MS (Link et al., 1973). Second, serum IgM antibodies to myelin oligodendrocyte glycoprotein (MOG) and myelin basic protein (MBP), the best candidate targets for demyelinating antibodies, were recently shown to have a predictive value indicating a new relapse in the initial phase of MS (Berger et al., 2003). MOG is the only myelin protein able to induce both encephalitogenic T cell responses and demyelinating antibodies (Lindert et al., 1999). However, new antibody specificities are being continuously discovered in MS, using recombinant proteins or new proteomic assays (Almeras et al., 2004). From another point of view, these methodologies may fail to expose relevant epitope structures, such as in oligosaccharide or in post-translationally modified antigens (Doyle and Mamula, 2001). This may be a reason for the discrepancies in results employing linear peptides and recombinant proteins versus natural antigens (Mantegazza et al., 2004).

We reported that glycopeptides were able to detect autoantibodies in MS (Mazzucco et al., 1999; Carotenuto et al., 2001), demonstrating, for the first time, the possibility of using a glycosylated peptide to identify autoantibodies in humans. The data further suggest the use of modified peptides as synthetic antigens in solid-phase ELISA to expose a conformational structure to the antibodies (Carotenuto et al., 2001). We developed a synthetic glycopeptide, called CSF114(Glc), able to optimize the recognition of antibodies in MS patient serum (Papini et al., 2002). In the present study, we report the occurrence of serum antibodies to CSF114(Glc) (IgM class, IgG class and IgG subclasses) in a large group of MS patients and compared the results with other inflammatory/ infectious nervous system disorders and other autoimmune diseases. The results obtained in MS were also correlated to disease duration, disability, different clinical course of the disease (i.e., relapsing versus progressive forms of MS) and treatments.

2. Materials and methods

2.1. Serum and cerebrospinal fluid collection

Serum was obtained for diagnostic purposes between 1998 and 2003 from patients and healthy BDs who had given their informed consent, and stored at -20 °C until use. Paired samples of CSF and serum were obtained from a group of MS patients after a diagnostic lumbar puncture.

2.2. Patients

The selected MS patients ($n = 186$), from different MS centers, fulfilled established international diagnostic criteria (Poser et al., 1983). The age of onset was between 10 and 50 years. The clinical findings, the EDSS and the clinical course of MS (Kurtzke, 1983; Lublin and Reingold, 1996) were evaluated at the time of blood sampling. CSF and MRI examinations were performed for diagnostic purposes. All patients received a course of steroids during relapse. The demographic and clinical characteristics of these patients are shown in Table 1.

As the control, we employed 166 blood donors (BDs), 25 patients affected by meningitis/encephalitis with a typical CSF pleocytosis used as an inflammatory neurological disease (IND) control and, as a control for other autoimmune diseases, we employed 41 patients affected by systemic lupus erythematosus (SLE) and 49 patients affected by rheumatoid arthritis (RA).

We detected the subclass restriction of IgG in patients presenting increased levels of IgG antibodies to CSF114(Glc). For this purpose, we selected 14 MS, 3 IND and 13 BD subjects with different levels of increased IgG antibody absorbance to CSF114(Glc).

Table 1
Demographic and clinical characteristics of 186 MS patients

Age, range (median)	17–69 (39)
Females, number (%)	156 (84%)
EDSS, range (median)	0–8.5 (3)
Disease duration, range (median)	1 months–34 years (9 years)
Patients in relapse[a]	42 (22%)
Clinical form:	
Relapsing/Remitting	156 (84%)
Secondary progressive	21 (11%)
Relapsing progressive	8 (4%)
Primary progressive	1 (1%)
Treatments:	
None	47 (25%)
Interferon-β, glatimer acetate or azathioprine	139 (75%)
Treatment duration, range (median)	15 days–4 years (1.5 years)

[a] A relapse was recorded within the 3 months preceding the blood sampling.

The antibodies were also measured in paired samples of CSF and serum obtained from a different group of 34 RR MS patients.

2.3. Preparation of CSF114(Glc)

CSF114(Glc) is a synthetic 21 amino acid residue glycopeptide, bearing a glucosyl moiety N-linked to an Asn residue. The sequence of the peptide and its specific glycosylation pattern (Papini et al., 2003) were selected from focused libraries of synthetic glycopeptides on the basis of their ability to specifically recognize autoantibodies in MS patient sera, using an immunoenzymatic assay. The glycopeptides were prepared by solid-phase peptide synthesis and purified to homogeneity by preparative high-pressure liquid chromatography (HPLC). The identity and the purity of the final product were checked by mass spectrometry and analytical HPLC (Papini et al., 2002).

2.4. Determination of serum antibodies by ELISA

Ninety-six well activated polystyrene ELISA plates (Limbro Titertek, ICN Biomedicals, Inc., Aurora, Ohio, USA) were coated with 1 μg/well of CSF114(Glc) in pure carbonate buffer 0.05 M (pH 9.6) and incubated at 4 °C overnight. After 5 washes in an automated ELISA washer (Columbus, Tecan, Austria) with saline containing 0.05% Tween 20, the plates were blocked by 10% fetal calf serum (FCS) in saline Tween (100 μl/well) at room temperature (RT) for 60 min. Sera diluted 1:100 and 1:2000 were applied at 4 °C for 16 h in saline Tween 10% FCS (2 dilutions were tested for each individual serum). After 5 washes, 100 μl/well of alkaline phosphatase conjugated anti-human IgM or anti-human IgG Fab$_2$-specific affinity purified antibody (Sigma, I0759/I9885, Saint Louis, Missouri, USA), diluted 1:400 (anti-IgM) or 1:2000 (anti-IgG) in saline Tween/FCS were added. After 3 h incubation at RT and 5 washes, 100 μl of substrate solution consisting of 2 mg/ml of *p*-nitrophenylphosphate (pNPP tablets N2765, Sigma, Saint Louis, Missouri, USA) in 10% diethanolamine buffer was applied. The reaction was blocked after 30 min with 50 μl of 1 M NaOH and the absorbance read in a multi-channel ELISA reader at 405 nm (Sunrise, Tecan, Austria). ELISA plates, coating conditions, reagent dilutions, buffers and incubation times were tested in preliminary experiments. Each plate included at least 2 positive and 2 negative controls. Each serum was individually evaluated at 2 dilutions (1:100 and 1:2000) to control the parallelism and dilution of antibody absorbance between the reference positive samples and the unknowns. Subclass specific mouse anti-human IgG conjugates (IgG1 9052-04, IgG2 9060-04, IgG3 9210-04, IgG4 9200-04, Southern Biotech, Birmingham, Alabama, USA) were used (diluted 1:1000) to detect the IgG subclass specific response. The antibody levels revealed

by ELISA were expressed both as an absorbance value at a dilution of 1:100 and as a titer (sample dilution which reaches the average plus three standard deviations of blanks). The analytical variability of the assays was evaluated repeating the same test (IgM and IgG determinations) in the same run (4 sera, 20 determinations each) or in different experiments (4 sera, 10 experiments on different days). The within-assay and between-assay coefficients of variation (SE/mean) were below 10% (IgM) or below 5% (IgG).

The cut-off values, employed to separate the results between BD and MS samples, were set for the IgM class in a ROC analysis, comparing different cut-off values as sensitivity, specificity and likelihood ratios (Habbema et al., 2002). The IgM cut-off was set at a level (absorbance 0.7) maximizing sensitivity, specificity and likelihood ratios. The IgG cut-off value was arbitrarily set at absorbance 1. The CSF samples ($n = 34$) were tested at 1:2 and 1:20 dilutions and the intrathecal synthesis of the antibodies in the CSF was evaluated employing the antibody index (Reiber and Lange, 1991).

2.5. Statistical analysis

The descriptive statistical analysis, the heterogeneity tests, the difference between parametric data or categorical variables and the linear regression and correlations were evaluated using StatView or GraphPad Prism statistical analysis software. To test for the heterogeneity of the results in different MS groups, we employed the Bartlett test to compare the variances observed in different groups (sex, different clinical form of the disease, disease activity and therapy). Due to heterogeneity of the data, we employed a distribution independent analysis. Within-group comparisons were performed with the non-parametric Kruskal–Wallis one-way analysis of variance (ANOVA), employing the Dunn multiple comparison test for post-analysis group comparison. The differences between frequencies of abnormalities revealed in different diagnostic groups were analyzed employing the Fisher Exact Test. The significance of the *P* values was set at <0.05.

3. Results

3.1. IgM antibodies to CSF114(Glc) in serum

The heterogeneity Bartlett test showed that the IgM antibody absorbance to CSF114(Glc) was heterogeneous between the various diagnostic groups. We employed a distribution independent analysis to compare the antibodies between the groups. The IgM antibody absorbance to CSF114(Glc) was higher in MS (median 0.49) compared to the BD (median 0.27, Dunn test, $p < 0.001$), RA (median 0.38, $p < 0.001$) and SLE (0.26, $p < 0.01$) subjects. The

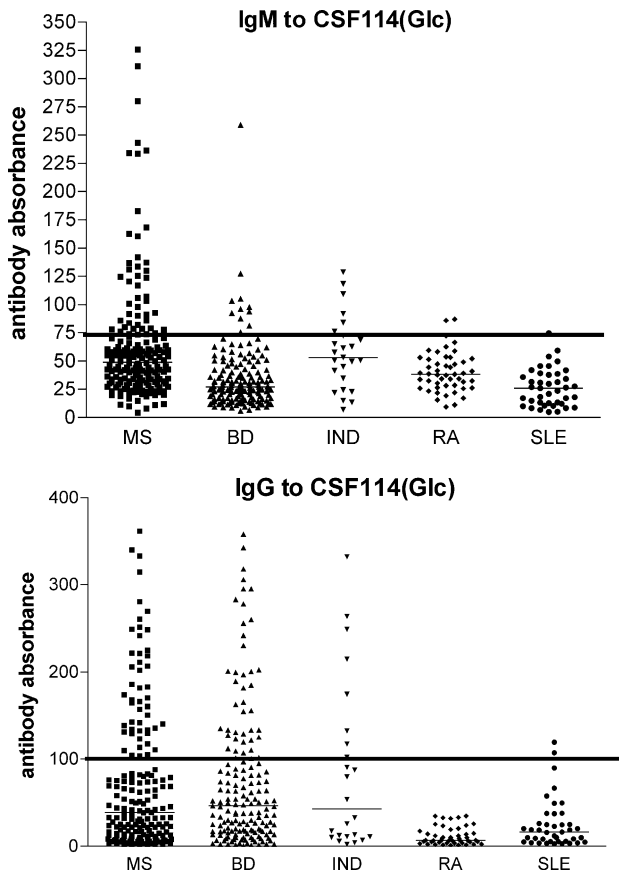Fig. 1. Column scatter and median values of IgM (upper panel) and IgG (lower panel) antibody absorbance ($\times 10^2$, dilution 1:100) to CSF114(Glc) in multiple sclerosis (MS, $n=186$), blood donors (BD, $n=166$), patients affected by meningitis/encephalitis (inflammatory neurological disease control, IND, $n=25$), systemic lupus erythematosus (SLE, $n=41$) and rheumatoid arthritis (RA, $n=49$). IgM antibody level to CSF114(Glc) is significantly increased in MS patients versus blood donors (Fisher Exact Test $p=0.005$) and versus RA ($p=0.001$) or SLE ($p<0.001$). The horizontal lines indicate the selected cut-off values for IgM and IgG determination and the median value of each group.

column scatter of the data is reported in Fig. 1 (upper panel). Although the differences between the MS and IND subjects were not statistically significant, the highest values of antibody absorbance (>1.3) were observed only in MS subjects. The ROC curve constructed to evaluate different cut-off values and separate the results observed between BD and MS subjects is reported in Fig. 2 (area under the curve 0.73, $p<0.03$). At an absorbance cut-off level of 0.7, we obtained a sensitivity of 26% (95% confidence interval (CI) between 20% and 32%), a specificity of 94% (89% to 97%) and a likelihood ratio of positives of 4.46 (95% CI 2.3 to 8.5). Considering the frequency of an increased IgM antibody level for CSF114(Glc) in the different diagnostic groups, the antibodies were detected in 50 MS patients (26%) versus 10 BDs (6%, Fisher Exact Test $p=0.005$). Compared to the BDs, increased levels were also found in 5/25 IND (20%) subjects, although only with lower absorbance values. No
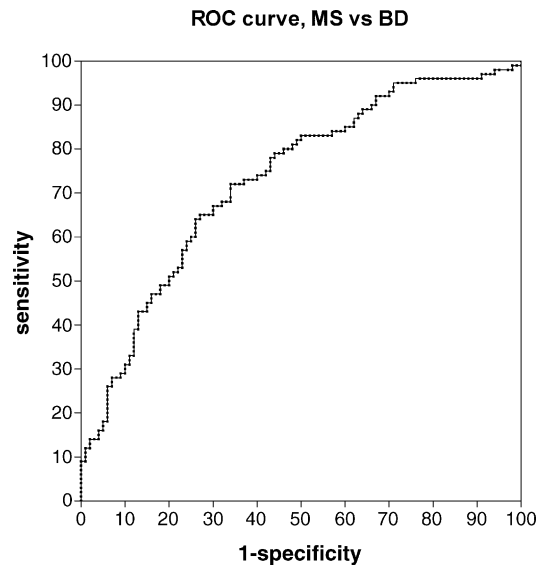

Fig. 2. ROC curve analysis of IgM antibodies to CSF114(Glc) in MS versus BD. The area under the curve is 0.73 ($p<0.03$). The cut-off point was set at an absorbance of 0.7 with a sensitivity of 26%, a specificity of 94%, and a positive likelihood ratio of 4.46.

IgM antibodies to CSF114(Glc) were detected in patients with RA (5/49) or SLE (1/41).

3.2. IgG antibodies to CSF114(Glc) in serum

The levels of IgG absorbance are reported in Fig. 1 (lower panel). The differences between MS and BD or the other groups were not significant.

3.3. IgM and IgG antibodies to CSF114(Glc) in CSF

The IgM antibodies to CSF114(Glc) were not detectable in the CSF (absorbance <0.04). The IgG antibodies in CSF were detected in 5/36 patients presenting the highest levels of IgG absorbance to CSF114(Glc) in serum, with no evidence of intrathecal synthesis of the antibodies, according to the antibody index formula.
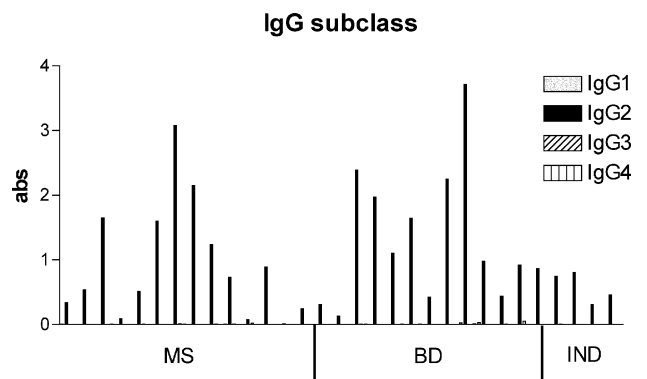

Fig. 3. IgG1, IgG2, IgG3 and IgG4 antibody absorbance to CSF114(Glc) in 14 MS, 13 BD and 3 IND subjects presenting the IgG antibody. All of the antibody reactivity is restricted to IgG2.

3.4. IgM and/or IgG antibodies to CSF114(Glc) in serum

A total of 83 MS patients (44%) presented IgG and/or IgM antibodies to CSF114(Glc) compared to 42 BDs (18%, Fisher Exact Test, $p < 0.0002$).

3.5. IgG 2 subclasses in MS and BD subjects

In 14 MS, 13 BD and 3 IND subjects, selected for presenting IgG antibodies, the antibodies were entirely restricted to the IgG2 subclass (Fig. 3).

3.6. Correlation to clinical variables

The heterogeneity Bartlett test showed that the IgM antibodies to CSF114(Glc) as well as the EDSS score and duration were not homogeneous in RR versus progressive MS patients, while no differences were detectable in relation to sex, disease clinical activity or therapy.

Evaluating the results as frequency of antibody reactivity, IgM positives to CSF114(Glc) were found in the serum of 46/156 (30%) RR MS patients compared to 2/21 (9%) secondary progressive patients only (Fisher Exact Test, $p = 0.07$). No significant differences were detected in relation to disease clinical activity or therapy.

When correlating the antibody reactivity to CSF114(Glc) and disease duration or disability (Fig. 4), the highest

Fig. 4. Correlation between IgM absorbance ($\times 10^2$, dilution 1:100) in serum to CSF114(Glc), disease duration (upper panel) and EDSS disability score (lower panel) in 156 relapsing/remitting MS patients. The linear correlation between increased IgM levels and disease duration or EDSS score is not significant.

antibody levels were found in RR patients with an EDSS score between 0 and 4 and a disease duration between 5 and 15 years. However, no significant linear correlation could be revealed between the antibodies and disease duration or EDSS scores.

4. Discussion

After intensive research, the targets of the putative B cell autoantibodies in MS are growing in numbers (Cross et al., 2001), and involve multiple structures in individual patients. It is possible that the pathogenic responses are hidden by irrelevant ones (Mathey et al., 2004). We considered that oligosaccharides are key epitopes in a number of clinically important antigenic structures, such as those in blood group determinants, xenotransplantation antigens or tumors, but have not yet been adequately studied in MS (Delves, 1998). We applied a chemical approach to the synthesis of a highly reproducible glycopeptide structure, optimized in parallel to an immunological detection using ELISA, able to reveal antibodies in MS that recognize a specific conformational epitope (Craig et al., 1998; Dharmasaroja, 2003). This enabled the reproducible and effective detection of IgM autoantibodies to glycopeptide CSF114(Glc) in a significant proportion (30%) of the RR MS patients, and with a high specificity for this disease (94%), i.e., no antibody reactivity was detected in other autoimmune diseases or other infective neurological diseases. Some patients with meningitis or encephalitis presented increased levels of antibodies. However, it should be considered that this result is not surprising as these diseases are usually employed as a control for autoimmune reactivity in serum during active acute neuro-inflammation and demyelination. This latter condition was already clearly associated to the activation of the autoantibody response to various myelin, viral, and bacterial antigens (Vartdal et al., 1980). The IgM reactivity to CSF114(Glc) in patients with meningo-encephalitis was, however, lower than in MS, confirming the specificity of the increased antibodies for MS.

Studying the clinical correlation of autoantibodies is an important step in characterizing the possible pathogenicity of the autoimmune response. Although the clinical/immunological correlations are difficult to detect in the cross-sectional studies (Karni et al., 1999), the antibodies detected in the present study were typical of RR patients, with a tendency toward the highest antibody absorbance after a disease duration between 5 and 15 years and with a low-medium disability, a disease phase associated to a predominant inflammation-demyelination within the nervous system (Lassmann, 2002; Compston and Coles, 2002). From a different point of view, IgM to myelin components may also be considered as having a possible protective role (Rosenbluth et al., 2003). No significant alterations were revealed in relation to disease activity and treatments.
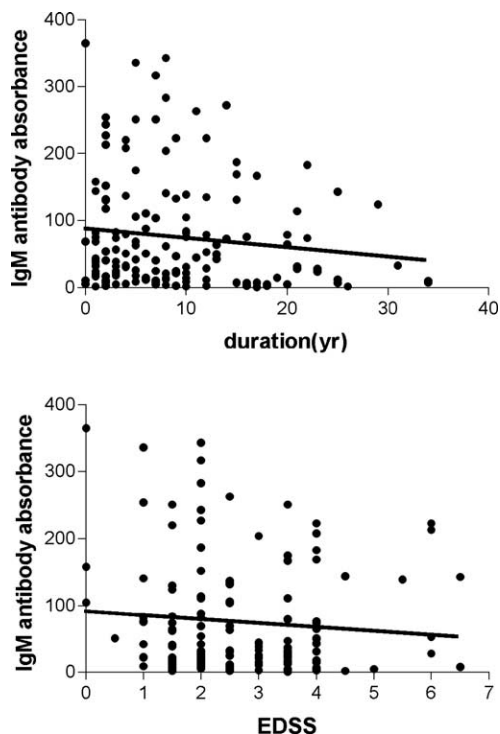
However, the majority of MS patients are presently subjected to a heterogeneous set of immunomodulating/immunosuppressive treatments and steroidal pulses, while only a few patients are left untreated, and these differences are difficult to detect.

The IgG antibodies produced in MS are usually restricted to IgG1 and IgG3 subclasses (Vandvik et al., 1976). In contrast with these previous results and as expected from the chemical structure of the antigen, the response to CSF114(Glc) was limited to the IgG2 subclass, a subclass characteristic of a T cell independent response to glycoepitopes and rarely found, until now, in MS (Garcia-Merino et al., 1986; Mathiesen et al., 1989; Egg et al., 2001). From this point of view, bacterial products, such as those containing polysaccharides, were detected in brain lesions and linked to B and T cell stimulation (Schrijver et al., 2001) and activation of inflammatory responses (Matyszak, 1998). In addition, it has already been shown that B cell activation in MS may be dependent on bacterial as well as viral antigens (Persson et al., 1989). An increasing number of mimicries between bacterial proteins and myelin antigens have been documented, suggesting a pathogenetic link between the antibody responses to bacterial carbohydrate products and those to myelin antigens (Hughes et al., 2003; Yuki et al., 2004). From another point of view, as glycosylation changes occur in different autoimmune diseases, many studies have recently focused on the role of immune responses to modified structures in autoimmune diseases and MS (Doyle and Mamula, 2001). An alternative hypothesis is based on the immune recognition of an altered brain glycoprotein. The difficulties of detecting an intrathecal response to CSF114(Glc) seem to contradict this hypothesis and further studies are needed to investigate the possibility of an antibody-mediated demyelination.

We should acknowledge that antibodies to myelin antigens or glycoepitopes are difficult to detect, are not consistently found in MS, and there are significant discrepancies between studies, particularly when taking into account the efforts needed for result standardization between laboratories (Mantegazza et al., 2004; Lampasona et al., 2004). Our results indicate, for the first time, the possibility of identifying a subgroup of MS patients with systemic antibody synthesis and a high specificity of response for MS. The discovery of an antibody response in MS specifically involving the IgM class is not surprising. IgM antibodies to oligodendrocyte structures were recently found to bind to glial cell membranes and induce calcium signaling. The biological importance of IgM reacting to different glycoepitopes was further confirmed in an experimental model in which the antibodies are synthesized within the CNS and play a different and contrasting role in demyelination versus remyelination "in situ" (Rosenbluth et al., 2003). The exact definition of the antibody epitope, clarification of the antigens inducing the response, and a longitudinal study of B cell activation to the glycosylated epitope of CSF114(Glc) in individual patients will clarify the issue on the relevance and prognostic value of the antibodies to CSF114(Glc) in the follow-up of MS.

5. Disclosures

Acknowledgement

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