Antibody responses against galactocerebroside are potential stage-specific biomarkers in multiple sclerosis

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Background: Galactocerebroside, the major glycolipid of central nervous system myelin, is a known target for pathogenic demyelinating antibody responses in experimental allergic encephalomyelitis (EAE), the animal model of multiple sclerosis (MS).

Objective: To address the importance of anti-galactocerebroside (α-GalC) antibodies in MS and to evaluate them as biomarkers of disease.

Methods: α-GalC IgGs were quantified from sera of patients with MS and in marmoset EAE by a new immunosorbent assay.

Results: We report a significant difference in serum α-GalC IgG titers between patients with relapsing-remitting (RR)–MS and healthy controls (HCs; $P < .001$). The frequencies of α-GalC antibody-positive subjects (α-GalC titers ≥ mean HC titers + 3 SD) are also significantly elevated in RR-MS compared with HC (40% vs 0%; $P = .0033$). Immunoaffinity purified α-GalC IgGs from human serum bind to cultured human oligodendrocytes, indicating that the ELISA detects a biologically relevant epitope. Corroborating these findings, α-GalC antibody responses in marmoset EAE were similarly found to be specifically associated with the RR forms and not the peracute or progressive forms, in contrast with other anti-myelin antibodies ($P = .0256$).

Conclusion: (1) α-GalC antibodies appear MS-specific and are not found in healthy subjects, unlike antibodies against myelin proteins; (2) when present, α-GalC antibodies identify mostly RR-MS and may be an indicator of ongoing disease activity. This novel assay is a suitable and valuable method to increase accuracy of diagnosis and disease staging in MS.

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Key words: *Galactocerebroside, myelin antigens, autoantibody, multiple sclerosis, experimental allergic encephalomyelitis*

Multiple sclerosis (MS) is a chronic immune-mediated inflammatory demyelinating disease of the central nervous system (CNS) characterized by heterogeneity in clinical presentation and underlying pathological mechanisms.[1] There is currently no easy paraclinical marker to diagnose MS subtypes and predict disease course accurately without lengthy periods of clinical follow-up.

Several myelin autoantigens may serve as targets for the autoaggressive attack in MS—for example, myelin protein myelin/oligodendrocyte glycoprotein (MOG), expressed on the outermost lamellae of the myelin sheath and thus readily accessible to the immune machinery; and a major CNS myelin glycolipid, galactocerebroside (GalC), which accounts for 32% of the myelin lipid content. Both MOG and galactocerebroside are highly encephalogenic in various models of experimental autoimmune encephalomyelitis (EAE), the prototypic animal model for MS.[2-4] Furthermore, passive antibody transfers in myelin basic protein (MBP)–primed animals[5-9] and *in vitro* models have demonstrated the demyelinating properties of anti-galactocerebroside (α-GalC) and α-MOG antibodies.[10-13] Antibody responses against these myelin targets are thus factors that potentially regulate

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Abbreviations used

AM: Acute monophasic
CIS: Clinically isolated syndrome
CNS: Central nervous system
EAE: Experimental allergic encephalomyelitis
GalC: Galactocerebroside
α-GalC: Anti-galactocerebroside
HC: Healthy control
HR: Hazard ratio
MBP: Myelin basic protein
MOG: Myelin/oligodendrocyte glycoprotein
MRI: Magnetic resonance imaging
MS: Multiple sclerosis
PP: Primary-progressive
rMOG: Recombinant rat myelin/oligodendrocyte glycoprotein (extracellular domain)
RR: Relapsing-remitting
RT: Room temperature
SP: Secondary-progressive

disease phenotype expression in the context of established CNS inflammation.

The pathogenic involvement of anti-myelin antibodies in human MS is less well established, because antibody titers against the myelin proteins do not unequivocally differ between control populations and patients with MS.[14-19] However, regardless of pathogenicity, anti-myelin antibodies have recently been proposed as predictive disease markers.[20]

Here, we examined whether α-GalC antibodies could serve as disease markers in MS. We demonstrate for the first time that significantly elevated titers of α-GalC antibodies are specifically found in relapsing-remitting (RR)–MS, and not in early or progressive forms of the disease. In strong support of our clinical observations, longitudinal assessment of galactocerebroside reactivity during the course of relapsing EAE in marmosets indicates that appearance of antibodies against galactocerebroside is delayed with respect to disease onset.

METHODS

Patients and controls

Sixty-five consecutive patients seen in our MS center, 51 meeting the diagnostic criteria for clinically definite MS,[21] were recruited for this study: 20 with RR-MS, 15 secondary-progressive (SP)–MS, and 16 primary-progressive (PP)–MS (Table I). In addition, 14 patients had a clinically isolated syndrome (CIS), ie, a single clinical attack suggestive of CNS demyelination. Twenty volunteers served as healthy controls (HCs). Both untreated patients and patients treated with IFN-β and glatiramer acetate were included in this study, but those treated with glucocorticoids within 3 months or on immunosuppressive therapy within 6 months of phlebotomy were excluded. Blood was drawn by venipuncture and clotted serum stored at −40°C. Informed consent was obtained from the patients and HCs, and the study was conducted in accordance with Institutional Review Board approval.

Animals

Callithrix 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 human white matter homogenate as described.[22] Plasma samples were obtained from EDTA-anticoagulated blood at baseline and at intervals of 2 to 4 weeks and stored at −40°C. The animals were scored every other day for the development of clinical signs and disability using a previously published scale.[22]

α-GalC ELISA

Bovine brain–derived galactocerebroside (Matreya, Pleasant Gap, Pa) was dissolved in chloroform-methanol (2:1). For coating, galactocerebroside was air-dried, stepwise resuspended in 65°C hot ethanol (50% vol/vol) at a final concentration of 50 ug/mL, with 100 uL added to wells of Polysorb 96-well microtiter plates (Nunc, Rochester, NY), and incubated uncovered overnight at room temperature (RT) for solvent evaporation. Plates were washed with double-distilled H_2O and blocked with 1% BSA (A7030; Sigma, St Louis, Mo) in PBS (ELISA buffer) for 2 hours at RT. After washing with PBS and ddH_2O, 100 uL of either human serum samples, diluted 1:40 in ELISA buffer, or *C jacchus* samples, diluted 1:100, were incubated in triplicate overnight at 4°C. Background binding of each sample was controlled for on blocked wells without coated antigen. After washing, specific antibody binding was detected by an alkaline phosphate–labeled goat-anti-human IgG (A9544; Sigma) or by a horseradish peroxidase–conjugated rabbit-anti-monkey IgG (A2054; Sigma), diluted in ELISA buffer and incubated for 1 hour at RT. For human sera, binding was detected by reading the OD at 405 nm in a microplate reader (SpectraMax; Molecular Devices, Sunnyvale, Calif) after incubation with paranitrophenyl phosphate (Moss, Pasadena, Md) for 30 minutes in the dark at RT. The marmoset assay was developed with 3,3′,5,5′-tetramethylbenzidine (Pierce, Rockford, Ill) for 15 minutes at RT and the OD read at 450 nm wavelength.

For specificity and sensitivity controls, a polyclonal rabbit-anti-bovine galactocerebroside antiserum (G9152; Sigma) was used and antibody binding detected by a horseradish peroxidase–labeled goat-anti-rabbit IgG (A0545; Sigma). Quenching experiments were performed by overnight pre-incubation with solubilized galactocerebroside; galactocerebroside was air-dried and resuspended in 65°C hot ethanol at 200 ug/mL and further diluted in ELISA buffer to a final concentration of 2 ug/mL.

Anti-myelin protein antibody ELISA

C jacchus antibodies against human MBP and recombinant rat (r)MOG, amino acids 1-125[23] were coated to microtiter plates (Maxisorb; Nunc) overnight with 1 ug antigen per well. After washing and blocking with 3% BSA in PBS plus .05% Tween for 1 hour at 37°C, marmoset samples were incubated for 1 hour at 37°C and diluted 1:100 in 3% BSA in PBS plus .05% Tween. Antibody binding was detected by a peroxidase-labeled rabbit-anti-monkey IgG for 1 hour at 37°C.

Statistical analysis

To express the results of the galactocerebroside assay, a signal-to-background binding ratio was calculated as the ratio of OD (signal) over OD (background). Positive controls, ie, a human sample with strong binding signal, and negative controls, ie, ELISA buffer only, omitting serum, were included on each plate. For human samples, samples above the mean binding ratio + 3 SD for the HC group were considered positive. In the marmoset assay, samples were considered positive for a binding ratio above 3 with OD_{GalC} >0.1 and greater than 3-fold the baseline (unimmunized) sample. Statistical