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Increased Circulating T Cell Reactivity to GM3 and GQ1b Gangliosides in Primary Progressive Multiple Sclerosis

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Abstract

We have previously shown that patients with primary progressive multiple sclerosis (MS) have significantly elevated plasma levels of antibody to GM3 ganglioside compared to patients with relapsing-remitting MS, healthy subjects and patients with other neurological diseases. Anti-GM3 antibody levels were elevated also in patients with secondary progressive MS but to a lesser extent than in primary progressive MS. As gangliosides are particularly enriched in the axonal membrane, these findings suggested that antiganglioside immune responses might contribute to the axonal damage in progressive forms of MS. The present study was performed to determine whether peripheral blood T cell responses to GM3 are also increased in progressive MS. Blood was collected from 98 untreated patients with MS (40 with relapsing-remitting, 27 with secondary progressive and 31 with primary progressive MS), 50 healthy subjects and 24 patients with other disorders of the CNS, and reactivity to GM1, GM3, GD1a, GD1b, GD3, GT1b, GQ1b and sulphatide was assessed by 6-day T cell proliferation assays. Increased T cell reactivity to GM3 and GQ1b occurred significantly more often in patients with primary progressive MS than in healthy subjects and patients with other CNS diseases. These findings suggest that ganglioside-specific T cells may contribute to the axonal damage in primary progressive MS.

Author Keywords: multiple sclerosis; gangliosides; T-lymphocytes; autoimmunity; disease progression

Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) and is a common cause of persistent disability in young adults. Typically MS has a relapsing-remitting course with clinical attacks followed by some improvement and attack-free intervals. Often, however, this initially relapsing-remitting course eventually changes to a progressive one in which progressive neurological deterioration occurs independently of relapses (secondary progressive MS). In 10–20% of cases the disease has a progressive course from onset (primary progressive MS). An immune attack directed primarily at axonal antigens rather than at myelin antigens could explain the progressive course of this form of MS. The inherent capacity for CNS axonal regeneration is much more limited than the capacity for CNS remyelination, which is likely to contribute to the clinical recovery from attacks of relapsing-remitting MS. It is also possible that the transition from relapsing-remitting to secondary progressive MS may involve the spreading of the immune response from myelin antigens to axonal antigens.[1]

Gangliosides constitute an important group of axonal antigens and are also minor constituents of myelin. They are particularly enriched in the plasma membranes of axons and neuronal cell bodies compared to the plasma membranes of most other cell types.[2] Gangliosides are glycolipids with one or more sialic acid residues in their oligosaccharide chains, and have been incriminated as potential target antigens in several diseases of the human peripheral nervous system. Convincing evidence that immune responses against gangliosides may indeed cause axonal degeneration has been provided by the induction of experimental sensory ataxic neuropathy in rabbits by immunization with GD1b, a ganglioside found in dorsal root ganglion neurons and dorsal root axons. [3]

Several studies have found increased antiganglioside antibody levels in the sera and/or cerebrospinal fluid (CSF) of patients with MS.[4, 5, 6, 7, 8, 9, 10, 11, 12, 13] Acarin et al. [11] and Sadatipour et al. [12] compared the levels among the subtypes of MS and showed increased antiganglioside antibody levels in patients with primary progressive MS. Several studies have also demonstrated increased peripheral blood T lymphocyte responses to mixed ganglioside preparations in patients with MS. [14, 15, 16, 17, 18] However, there have been no studies comparing T cell reactivity to individual gangliosides among the relapsing-remitting, secondary progressive and primary progressive forms of MS. The present study was performed to determine the T cell responses to individual gangliosides in the different forms of MS. We found increased peripheral blood T cell proliferative responses to GM3 and GQ1b gangliosides in patients with primary progressive MS.

Materials and methods

Patients and control subjects

The subjects of this study consisted of 98 patients with MS, 50 healthy control subjects and 24 patients with other CNS diseases. All the patients with MS met the criteria of Poser et al.[19] for clinically definite or laboratory-supported definite MS. All except 4 patients with MS also met the criteria of McDonald et al. [20] for MS; these 4

could not be assessed against these criteria because magnetic resonance imaging scans were not available for inspection. Of the MS patients, 40 had relapsing-remitting MS, 27 had secondary progressive MS and 31 had primary progressive MS, as defined by the criteria of Lublin and Reingold. [21] MS patients had not received corticosteroid or other immunomodulatory therapies for at least 2 months prior to being studied except for 1 patient who ceased interferon therapy 5 weeks before being studied. Patients with other CNS diseases had the following diagnoses: cerebral infarction (5), epilepsy with associated cerebral pathology (5), motor neuron disease (4), brain haemorrhage (3), spinal cord infarction/ischaemic myelopathy (2), intracranial meningioma (1), coeliac disease with progressive cerebellar ataxia (one), viral encephalitis (1), Krabbe leukodystrophy (1) and neurofibromatosis type I with optic nerve glioma (1). This study was approved by the Human Research Ethics Committee of the Royal Brisbane Hospital and the Medical Research Ethics Committee of The University of Queensland.

Tissue typing

Genomic DNA was prepared from either Epstein-Barr-virus transformed lymphoblastoid cell lines or from heparinized whole blood by either chloroform/phenol extraction, or by overnight digestion using sodium dodecyl sulphate/proteinase K, followed by salting out of high molecular weight DNA. HLA-DRB1 tissue typing was performed using SSP HLA typing kits (Dynal Biotech, Oslo, Norway).

Antigens

Bovine gangliosides GM1, GM3, GD1a, GD1b, GD3, GT1b and GQ1b and sulphatide were purchased from Sigma (St. Louis, USA). Their structures are shown in Fig. 1. To determine the optimal concentration of ganglioside for use in the proliferation assays, cells from 20 patients with MS and 20 healthy subjects were initially tested in culture at concentrations ranging from 800 to 0.005 ng/ml.

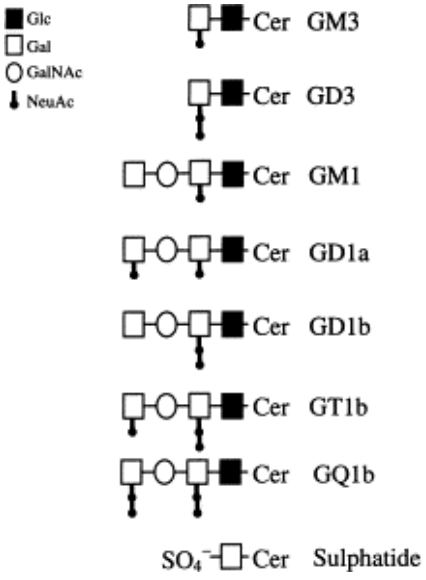


Fig. 1. Structures of gangliosides and sulphatide used in this study. Glc, glucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; NeuAc, *N*-acetylneuraminic acid; cer, ceramide.

Proliferation assays

Heparinized peripheral blood (~60 ml) was collected by venepuncture from each subject after informed written consent had been obtained. Peripheral blood mononuclear cells were separated from blood by centrifugation through Histopaque (Sigma Chemical, St. Louis, USA) and washed twice. Proliferation assays were prepared as 200 μl/well, 10^5 cells/well quadruplicate cultures with and without test antigens in 96-well round-bottomed microtitre plates (Nunc, Denmark) in RPMI-1640 media supplemented with 10% heat-inactivated pooled human serum, 2 mM L-glutamine and 10 mM HEPES buffer. Cultures were incubated for 6 days, with 0.5 μ Ci [^{3}H]thymidine being added during the last 18 h. Cultures were harvested and thymidine uptake was measured in c.p.m. (counts per minute) in a β plate counter (LKB). For each ganglioside tested, quadruplicate cultures were prepared against a range of 5 ganglioside concentrations. These concentrations were 1, 0.1, 0.05, 0.01 and 0.005 ng/ml. The stimulation index (SI) was determined by the formula: SI=(mean c.p.m. of quadruplicate, ganglioside-containing wells)/(mean c.p.m. of 24 control wells, without antigen). A positive proliferative response for a test subject's cells was scored if the cells responded to the ganglioside tested at any 1 of the 5 concentrations with an SI≥2.0.

Statistical analysis

Percentages of individuals making a positive proliferative response to gangliosides were compared using the χ^2 test with Yates' correction applied as required. Mean SI values were compared using analysis of variance (ANOVA) to compare all the test groups simultaneously, followed by Student's *t* test to compare pairs of groups. A comparison was deemed to show statistical significance if $P \leq 0.05$.

Results

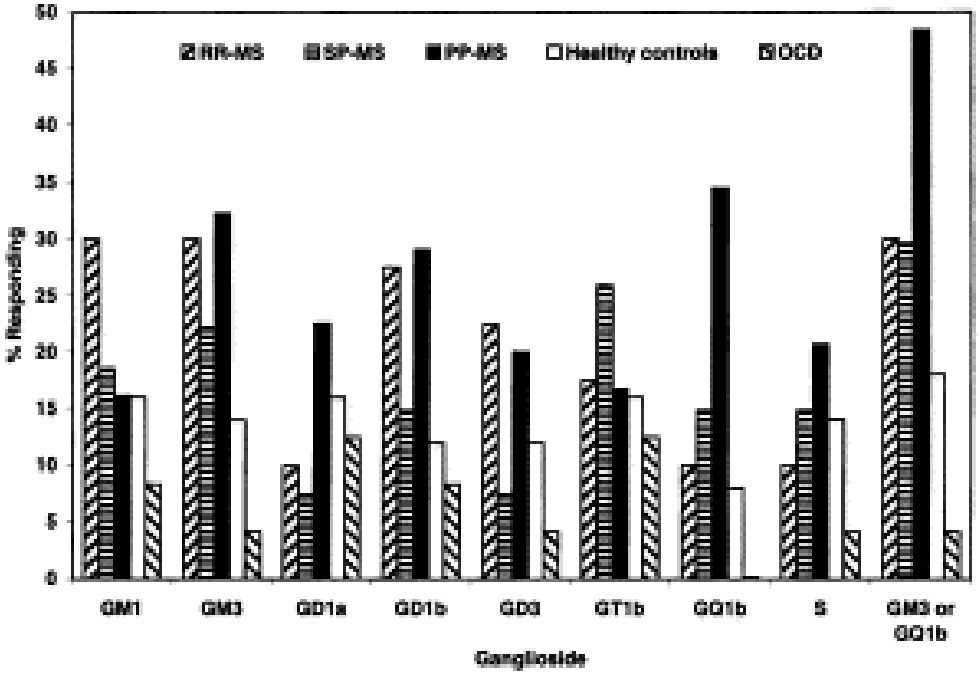
The characteristics of the patients with MS, the healthy subjects and the patients with other CNS diseases are shown in Table 1. The percentages of individuals in each subject group with a SI≥2.0 were determined for each ganglioside and compared by χ^2 analysis (Fig. 2). The percentage of patients with primary progressive MS with a SI≥2.0 for GM3 (32.3%) was significantly higher than the percentages of healthy subjects (14.0%) and patients with other CNS diseases (4.2%), but the percentage for patients with secondary progressive MS (22.2%) was not. The corresponding percentage for patients with relapsing-remitting MS (30.0%) was significantly higher than for patients with other CNS diseases but not significantly higher than for healthy subjects. As a combined group, patients with MS had a significantly higher percentage of individuals with a SI≥2.0 for GM3 (28.6%) than did healthy subjects (P=0.048) or patients with other CNS diseases (P=0.024). For GQ1b, the percentage of patients with primary progressive MS with a SI≥2.0 (34.5%) was also significantly higher than the percentages of healthy subjects (8.0%), patients with other CNS diseases (0.0%) and patients with relapsing-remitting MS (10.0%; P=0.028), but the corresponding percentages for patients with relapsing-remitting MS (10.0%) and secondary progressive MS (14.8%) were not significantly increased. Increased T cell reactivity (SI≥2.0) to GM3 or GQ1b occurred significantly more often in patients with primary progressive MS (48.4%) than in healthy subjects (18.0%) and patients

with other CNS diseases (4.2%). T cell reactivity to the other gangliosides was not significantly increased in any of the three groups of MS patients.

Table 1. Characteristics of MS patients and control subjects

	Number (female:male)	Mean age in years (range)
MS (whole group)	98 (72:26)	46.3 (18–77)
RR-MS	40 (30:10)	37.0 (18–60)
SP-MS	27 (21:6)	52.0 (36–72)
PP-MS	31 (21:10)	53.0 (29–77)
Healthy controls	51 (33:18)	31.6 (22–72)
OCD	24 (6:18)	50.3 (23–69)

RR-MS: relapsing-remitting MS, SP-MS: secondary progressive MS, PP-MS: primary progressive MS, OCD: other CNS diseases.



	GM1	GM3	GD1a	GD1b	GD3	GT1b	GQ1b	S	GM3 or GQ1b
5 x 2	0.244	0.041	0.538	0.104	0.175	0.789	0.002	0.539	0.003
RR-MS vs HC	0.112	0.064	0.603	0.061	0.184	0.824	0.966	0.801	0.181
SP-MS vs HC	0.970	0.358	0.474	0.996	0.811	0.293	0.586	0.807	0.240
PP-MS vs HC	0.767	0.049	0.458	0.055	0.331	0.841	0.007	0.439	0.004
RR-MS vs OCD	0.085	0.030	0.917	0.127	0.109	0.858	0.286	0.718	0.030
SP-MS vs OCD	0.517	0.143	0.869	0.778	0.816	0.384	0.149	0.421	0.044
PP-MS vs OCD	0.650	0.024	0.542	0.117	0.188	0.965	0.004	0.173	0.001

Fig. 2. Percentages of patients with relapsing-remitting MS (RR-MS), patients with secondary progressive MS (SP-MS), patients with primary progressive MS (PP-MS), healthy control subjects (HC) and patients with other CNS diseases (OCD) responding with SI$\geq$2.0 to specific gangliosides. The *P* values for the comparison of the responses of the five groups together (5×2) and the *P* values for the comparisons of the pairs of groups (χ^2 analysis) are shown directly below the ganglioside to which they refer. S, sulphatide.

The mean maximum SI±SE for GM3 and GQ1b for each subject group is shown in Table 2. For GM3 the mean maximum SI for patients with primary progressive MS

(2.05±0.30) was significantly higher than for healthy subjects (1.45±0.11) and for patients with other CNS diseases (1.27±0.09); it was also significantly higher for patients with relapsing-remitting MS (1.87±0.20) and for patients with secondary progressive MS (1.71±0.19) than for patients with other CNS diseases, but not significantly higher than for healthy subjects. As a combined group, patients with MS had a significantly higher mean maximum SI for GM3 (1.88±0.13) than did healthy subjects (P=0.035) and patients with other CNS diseases (P=0.025). For GQ1b, the mean maximum SI for patients with primary progressive MS (1.97±0.33) was significantly higher than for healthy subjects (1.36±0.11) and for patients with relapsing-remitting MS (1.31±0.10) but not significantly higher than for patients with other CNS diseases (1.25±0.08). HLA-DR typing of the patients with primary progressive MS did not reveal any association between T cell reactivity to GM3 or GQ1b and HLA-DR type.

Table 2. Mean maximum stimulation index (SI±SE) of peripheral blood mononuclear cells from relapsing-remitting MS (RR-MS) patients, secondary progressive MS (SP-MS) patients, primary progressive MS (PP-MS) patients, healthy controls (HC) and patients with other CNS diseases (OCD)

	GM3	GQ1b
RR-MS	1.87 ± 0.20	1.31 ± 0.10
SP-MS	1.71 ± 0.19	1.44 ± 0.14
PP-MS	2.05 ± 0.30	1.97 ± 0.33
HC	1.45 ± 0.11	1.36 ± 0.11
OCD	1.27 ± 0.09	1.25 ± 0.08

RR-MS vs HC: 0.043; RR-MS vs OCD: 0.025; SP-MS vs HC: 0.026; SP-MS vs OCD: 0.029; PP-MS vs HC: 0.035; PP-MS vs OCD: 0.042

ANOVA was used to compare RR-MS patients, SP-MS patients, PP-MS patients, healthy controls and patients with other CNS diseases, simultaneously; for GM3, P=0.036: for GQ1b, P=0.029. Student's t test was used to compare pairs of groups; significant P values are given adjacent to brackets linking pairs.

Discussion

The cause of MS is unclear but there is increasing evidence that it is an autoimmune disease.[22, 23] We have previously shown that patients with relapsing-remitting or secondary progressive MS have increased peripheral blood T cell proliferative responses to two overlapping myelin proteolipid protein (PLP) peptides, $PLP_{184–199}$ and $PLP_{190–209}$.[24] This region of PLP is immunodominant in humans [25] and is encephalitogenic in mice of different genetic backgrounds. [26] Furthermore, longitudinal studies in these patients have shown an increased frequency of surges of circulating T cell reactivity to this region of PLP, which partly correlate with disease activity. [27] These findings suggest that the increased T cell response to the 184–209 region of PLP may be pathogenic in relapsing-remitting and secondary progressive MS. In contrast, patients with primary progressive MS did not have increased T cell reactivity to this region of PLP or to any of the other myelin antigens studied. [24] This raised the possibility that the main target antigens in primary progressive MS are not myelin antigens but are perhaps axonal antigens. [1]

The present study has shown that patients with primary progressive MS have increased circulating T cell reactivity to gangliosides GM3 and GQ1b. The increased immune reactivity to GM3 and GQ1b in primary progressive MS may contribute to axonal damage or may be secondary to it. The fact that the anti-GM3 and anti-GQ1b

immunoreactivity is significantly higher in patients with primary progressive MS than in patients with CNS axonal damage due to other neurological diseases indicates that the immunoreactivity is not secondary to the axonal damage and that it may be pathogenic. Indeed the T cell reactivity against gangliosides tended to be lower in patients with other CNS diseases than in healthy subjects, as we have previously reported for T cell reactivity to myelin basic protein.[24] It should be noted that the proportion of primary progressive MS patients with increased T cell reactivity to GM3 and/or GQ1b in the present study may be an underestimate, as it is possible that the level of anti-ganglioside T cell reactivity in MS may fluctuate, as we have previously demonstrated for T cell reactivity against the 184–209 region of PLP. [27] At present it is unclear whether the increased T cell reactivity to GM3 and GQ1b in primary progressive MS is directed to separate antigenic epitopes or to a common moiety present in GM3 and GQ1b and perhaps other unstudied gangliosides.

The paucity of recent studies of T cell reactivity against gangliosides in MS has probably been partly due to the lack of knowledge of the molecular mechanisms by which T cells recognize lipid and glycolipid antigens. This has been in striking contrast to the well understood presentation of peptide antigens to T cell receptors by major histocompatibility complex (MHC) class I and class II molecules. Recently, however, the mechanism of presentation of lipid and glycolipid antigens to T cells has been elucidated. These antigens are bound via their acyl chains in the deep hydrophobic groove of the MHC-like CD1 molecule so that their hydrophilic components are positioned for highly specific interactions with T cell antigen receptors.[28]

The increased T cell reactivity to GM3 fits with our previously reported finding of increased circulating anti-GM3 antibodies in primary progressive MS [12]; we did not measure anti-GQ1b antibodies in that study. The present study also suggests that patients with relapsing-remitting or secondary progressive MS have increased T cell reactivity to GM3, whereas our previous study found anti-GM3 antibodies to be normal in relapsing-remitting MS and increased in secondary progressive MS, although less so than in primary progressive disease. Acarin et al. [11] also found elevated serum antiganglioside (anti-GM1, anti-asialo GM1 and anti-GD1a) antibody levels significantly more frequently in patients with primary progressive MS than in those with relapsing-remitting MS or secondary progressive MS.

Definite evidence that autoimmune responses against gangliosides are pathogenic in human disease remains elusive, although there is much circumstantial evidence to support this proposal. For example, increased levels of anti-GM1 antibodies occur in patients with the Guillain–Barre syndrome subsequent to Campylobacter jejuni enteritis [29] and in those with multifocal motor neuropathy. [30] Anti-GQ1b antibodies are elevated in patients with the Miller Fisher syndrome of ophthalmoplegia, ataxia and areflexia, [31] and increased anti-GD1a antibodies are associated with the acute motor axonal neuropathy subtype of the Guillain–Barre syndrome. [32] Furthermore, the induction of experimental sensory ataxic neuropathy in rabbits by immunization with GD1b3 proves that antiganglioside responses can be pathogenic.

In conclusion, our present finding of increased circulating T cell reactivity to GM3 and GQ1b in primary progressive MS raises the possibility that ganglioside-specific T

cells may contribute to the pathogenesis of axonal damage in this form of MS.

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