Roles of complex gangliosides in the development of experimental autoimmune encephalomyelitis

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Received on September 11, 2007; revised on December 28, 2007; accepted on February 20, 2008

We induced experimental autoimmune encephalomyelitis (EAE) in GM2/GD2 synthase knockout mice (GM2/GD2−/−), which cannot synthesize complex gangliosides, such as GM1, GD1a, GD1b, GT1b, and GQ1b, to investigate the roles of complex gangliosides in the pathogenesis of this disease. We used myelin-oligodendrocyte glycoprotein (MOG) as an immunogen. In active immunization EAE, the severity of clinical score was not different but the disease onset was significantly delayed in GM2/GD2−/− compared with those in wild-type mice. When we transferred MOG-reactive T cells from GM2/GD2−/− or wild-type mice to wild-type mice, no significant differences were observed between the two groups. In contrast, when we transferred MOG-reactive T cells from wild-type mice to GM2/GD2−/− or to wild-type mice, the onset of EAE in GM2/GD2−/− mice was delayed. The recall response of MOG-specific T cells, the function of antigen presenting cells, or the expression of several adhesion molecules in the endothelium were not significantly different between GM2/GD2−/− and wild-type mice. On the other hand, quantitative analysis of cellular infiltration in the central nervous system (CNS) on day 9 of active immunization EAE showed that the CD4+ cell number in the CNS isolated from GM2/GD2−/− mice was significantly less than that from wild-type mice. It indicated that the delayed onset of EAE in GM2/GD2−/− mice was due to the delay of the migration of pathogenic T cells into the CNS. Thus, the complex gangliosides may be involved in the T cell–endothelial cell interaction in the pathogenetic process of EAE.

Keywords: EAE/MS/neuroimmunology/rodent/cell surface molecules

Introduction

Gangliosides are sialic acid-containing glycosphingolipids that are highly enriched in the mammalian nervous system. They consist of an oligosaccharide core structure with an attached sialic acid and ceramide and are found primarily in the outer leaflet of the cell membrane. Although they are the major sialo-glycoconjugates in the brain, their biological functions remain to be elucidated.

In human autoimmune neuropathies, such as Guillain-Barré syndrome and IgM paraproteinemic neuropathy, antibodies against gangliosides are present in the patients' sera (Chiba et al. 1992). They are considered as useful diagnostic markers and essential factors involved in the pathogenetic mechanisms (Obi et al. 1992). Gangliosides may therefore act as targets for autoimmune mechanisms in autoimmune neuropathies (Kusunoki et al. 1996). In contrast, the role(s) of the gangliosides in the pathogenesis of the immune-mediated diseases of the central nervous system (CNS) have not yet been elucidated.

Experimental autoimmune encephalomyelitis (EAE) is an immune-mediated encephalomyelitis that can be generated in experimental animals using myelin proteins as an immunogen. The roles of gangliosides in the pathogenesis of EAE remain controversial. It has previously been reported that sensitization with gangliosides does not induce EAE, whereas the administration of gangliosides with myelin proteins either enhances or suppresses the disease activities or has no effect (Kusunoki et al. 1988; Shimada et al. 1994; Saez-Torres et al. 1998). The presence of definite immune responses against gangliosides has not been shown previously. On the other hand, gangliosides on the neural, glial, or immune cell membranes might be required in the pathogenetic mechanisms of EAE, in particular in the process of lymphocyte activation, migration, and their cytotoxic activities against the CNS.

Mice engineered to lack a key enzyme in complex ganglioside biosynthesis (GM2/GD2 synthase), which consequently do not synthesize complex gangliosides, such as GM1, GD1a, GD1b, GT1b, and GQ1b, have been reported previously (Takamiya et al. 1996; Furukawa et al. 2002). These animals express only the simple ganglioside molecular species, such as GM3 and GD3. We induced EAE in this strain of mice, between 8 and 12 weeks of age, to investigate the roles of complex gangliosides in the pathogenetic mechanisms of EAE by examining any possible difference in the clinical phenotype, disease course, and immunopathological findings. Investigations of EAE in GM2/GD2−/− mice should give us a clue to elucidate the roles of complex gangliosides in the nervous system, as well as in the immune system.

Results

Disease onset of EAE was delayed in GM2/GD2−/− mice

To examine the role of complex gangliosides in the development of EAE, we first performed active immunization to establish EAE. As for the severity of EAE score, including maximum EAE score and cumulative score, there was no significant

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Table I. Statistical analysis of clinical EAE scores

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Incidence</th>
<th>Day of onset</th>
<th>Max. score</th>
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<tr>
<td>GM2/GD2−/−</td>
<td>18/21 (85.7%)</td>
<td>17.6 ± 0.9*</td>
<td>3.10 ± 0.31</td>
<td>47.9 ± 5.4</td>
</tr>
<tr>
<td>Wild-type</td>
<td>15/17 (88.2%)</td>
<td>12.8 ± 0.9</td>
<td>3.03 ± 0.34</td>
<td>50.4 ± 6.5</td>
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<td>0.97 ± 0.26</td>
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</tr>
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<td>0.97 ± 0.22</td>
<td>6.72 ± 1.76</td>
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<td></td>
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<td>GM2/GD2−/−</td>
<td>18/24 (75.0%)</td>
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(A) Active immunization EAE. Each mouse was immunized with MOG35−55 peptide for the induction of EAE. Data were the same as Figure 1. (B) Adoptive transfer EAE. One million encephalitogenic CD4+ T cells from wild-type mice or GM2/GD2−/− mice (donor) were injected into wild-type mice (i.v.). Data were the same as Figure 3A. (C) Another type of adoptive transfer EAE. One million encephalitogenic CD4+ T cells from wild-type mice were injected intravenously into wild-type mice or GM2/GD2−/− mice (recipient). Data were the same as Figure 3B.

Mean ± SEM of the following parameters are shown: maximum score of EAE (Max. score), the days of EAE onset, incidence of paralyzed mice among sensitized rats (Incidence), and summation of the clinical scores from days 0 to 30 (Cumulative score). The statistical significance of the difference was determined using ANOVA; *P < 0.05 versus wild-type mice.

Fig. 1. Disease onset of EAE is delayed in GM2/GD2−/− mice. EAE was induced in GM2/GD2−/− or wild-type mice by immunization with MOG35−55 in the CFA, as described in Materials and methods. Statistical analysis is shown in Table IA. One representative experiment of two independent experiments was expressed as the mean EAE score.

Fig. 2. Complex gangliosides play a role in the effector and in the induction phases of EAE. (A) Encephalitogenic T cells were prepared by immunizing wild-type mice or GM2/GD2−/− mice and culturing their lymph node cells in the presence of MOG and IL-12 for 4 days. One million CD4+ cells were injected into the tail vein of wild-type mice. EAE clinical scores were assessed as described in Table IB. (B) As described above, MOG-specific CD4+ cells from wild-type mice were transferred into wild-type mice or GM2/GD2−/− mice. EAE clinical scores were assessed as described in Table IC. These data are shown as a mean clinical score ± SEM. Data are representative of three independent experiments.

Fig. 3. Comparison of MOG35−55-specific T-cell response in GM2/GD2−/− and wild-type mice. Popliteal and inguinal lymph nodes cells from GM2/GD2−/− mice or wild-type mice were incubated in the presence of MOG35−55 for 48 h. The proliferative response was determined by the uptake of [3H] thymidine (A), and IFN-γ was detected by ELISA (B). Representative data of two independent experiments are shown (n = 5 for each group). Error bars represent SEM.

Complex gangliosides do not have an effect on the recall response of MOG-specific T cells

To determine the mechanisms of complex gangliosides in the T-cell activation, we examined the proliferative response of MOG-specific CD4+ T cells from wild-type mice or GM2/GD2−/− mice. Popliteal and inguinal lymph nodes cells from GM2/GD2−/− mice or wild-type mice were incubated in the presence of MOG35−55 for 48 h. The proliferative response was determined by the uptake of [3H] thymidine (A), and IFN-γ was detected by ELISA (B). Representative data of two independent experiments are shown (n = 5 for each group). Error bars represent SEM.

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Complex gangliosides do not have an effect on the recall response of MOG-specific T cells

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and choroid plexus in the brain with ICAM-1, VCAM-1, E-selectin, and P-selectin was not significantly different between GM2/GD2\(^{-/-}\) and wild-type mice. These findings suggested that complex gangliosides do not have an effect on the presentation of adhesion molecules via the BBB. In addition, we examined MCP-1, which is also an important chemokine for immigration of autoreactive cells into the brain, using ELISA. MCP-1 was detected in the serum from EAE mice at 7 and 10 days after induction of EAE. The amount of MCP-1 was almost the same between GM2/GD2\(^{-/-}\) and wild-type mice (data not shown).

Delayed infiltration of pathogenic T cells into the CNS in GM2/GD2\(^{-/-}\) mice
To confirm the delayed infiltration of pathogenic T cells into the CNS in GM2/GD2\(^{-/-}\) mice, we isolated mononuclear cells in the CNS obtained from each mouse on day 9 of active immunization EAE. It is the time just before the onset of EAE for wild-type mice. No mice actually showed EAE symptoms at that time. As shown in Figure 5, the CD4\(^+\) cell number in the CNS isolated from GM2/GD2\(^{-/-}\) mice was significantly less than the cell number from wild-type mice (P < 0.05 by the Mann–Whitney U-test).

Discussion
By the active immunization and the adoptive transfer experiment, we demonstrated that the lack of complex gangliosides delayed the disease onset of EAE.

There have been several reports describing the association of gangliosides and lymphocyte activation or differentiation (Rouquette-Jazdanian et al. 2005; Shen et al. 2005). Actually, the administration of gangliosides has been reported to have an effect on the immune system in animals affected with EAE. Suppression of EAE in Lewis rats by the administration of gangliosides has been reported. It was shown that gangliosides
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significantly suppressed myelin basic protein-induced proliferation in a dose-dependent manner (Rouquette-Jazdanian et al. 2005). Chronic relapsing-remitting EAE, induced with MOG15–55 in NOD mice, was successfully treated with brain-derived gangliosides. Splenocytes from the ganglioside-treated mice displayed markedly attenuated levels of MOG15–55-specific proliferation and IFN-γ production (Sekiguchi et al. 2001). These reports raise the possibility that gangliosides are incorporated into the cell membrane of the lymphocytes to suppress their activity and ameliorate EAE. However, in the present study, adoptive transfer of MOG-specific T cells from GM2/GD2−/− or wild-type mice showed no significant difference, indicating that the presence of complex gangliosides does not influence the activation of the T cells.

As for the immune system, the spleen and thymus are slightly smaller in GM2/GD2−/− mice compared with those in wild-type (Zhao et al. 1999). GM1, asialo-GM1, and GD1b were representative gangliosides expressed on T cells of the wild-type mice and were completely abrogated on those of the GM2/GD2−/− mice. Splenocytes from the GM2/GD2−/− mice showed clearly attenuated proliferation compared with the wild-type mice when stimulated by IL-2 but not when they were treated with concanavalin A or anti-CD3 cross-linking. Expression levels of IL-2 receptor alpha, beta, and gamma were almost equivalent, and upregulation of alpha chain after T-cell activation was also similar between the GM2/GD2−/− and wild-type mice. Activation of JAK1, JAK3, and SAT5 after IL-2 treatment was reduced, and c-fos expression was delayed and reduced in the mutant splenocytes, suggesting that the IL-2 signal was attenuated in the mutant mice, likely due to the modulation of the IL-2 receptors by the lack of complex gangliosides (Zhao et al. 1999).

In our study, the proliferative response of MOG15–55-specific T cells in GM2/GD2−/− mice did not decrease in comparison with that in wild-type mice. Thus, the attenuation of IL-2 signal due to the lack of complex gangliosides was not involved in the induction phase of EAE.

Gangliosides are known to activate microglia via mitogen-activated protein kinase and NF-κB (Pyo et al. 1999; Marconi et al. 2005). Microglias are the brain-resident macrophages and play a crucial role in EAE. GT1b can induce production of nitric oxide (NO), and tumor necrosis factor-alpha (TNF-α) and expression of cyclooxygenase-2 (COX-2). GM1 and GD1a are also able to induce expression of COX-2 (Pyo et al. 1999). These molecules are important for an inflammatory response in EAE. COX-2 is inductively expressed in inflammatory cells following an exposure to proinflammatory and mitogenic stimuli, and is primarily responsible for the synthesis of prostanoids involved in acute and chronic inflammation (Xie et al. 1991). We have reported that COX-2 inhibition is effective for the treatment of the autoimmune diseases, EAE and EAN (Miyamoto et al. 1998, 1999, 2002, 2006). Therefore, the lack of complex gangliosides may reduce an inflammatory response in the CNS due to inadequate activation of microglia.

Myelin-associated glycoprotein (MAG) and complex gangliosides contribute to axon stability in both the CNS and peripheral nervous system (PNS). Similar neuropathological and behavioral deficits in Galgt1−/−, Mag−/−, and double-null mice support the hypothesis that MAG binding to gangliosides contributes to a long-term axon–myelin stability (Pan et al. 2005). However, these neuropathological findings were not reported in the GM2/GD2−/− mice aged between 8 and 12 weeks. In addition, even if there was any instability in the nervous system of GM2/GD2−/− mice of this age range, it would likely have an enhancing effect on the development of EAE. It is therefore suggested that the latent instability of the nervous system is not the cause of the delay of the EAE onset observed in GM2/GD2−/− mice.

Cell surface gangliosides inhibit cellular immune responses, including APC development and function, which is critical for Th1 and Th2 cell development (Caldwell et al. 2003). APC activity is an important step in the cellular immune response. Gangliosides have the ability to interfere with a number of APC functions, including Ag processing and presentation, cytokine production, and induction of lymphocyte proliferation. For example, when human monocytes were preincubated with purified ganglioside GD1a, the expected Ag-induced proliferative response of autologous normal T cells added to these monocytes was inhibited. Upregulation of the monocyte costimulatory molecule, CD80, was almost completely inhibited (Caldwell et al. 2003). In a study using human dendritic cells (DCs) and naïve CD4+ T cells, preincubation with purified GD1a reduced the upregulation of costimulatory molecules and pertussis toxin-induced IL-12 production, whereas cholera toxin-induced IL-10 production was increased. Thus, ganglioside exposure of DCs could adversely affect the development of an effective cellular immune response (Shen et al. 2005). Therefore, the immune ability of APCs in GM2/GD2−/− mice could be more active than that in wild-type mice. However, in the present study, the clinical feature of EAE was the opposite; i.e., the EAE response was delayed in GM2/GD2−/− mice. Furthermore, there was no significant difference in APC function between GM2/GD2−/− and wild-type mice in an in vitro study. This suggested that complex gangliosides do not affect the APC function in the development of EAE.

It is also possible that the migration of the activated lymphocytes is affected by the lack of complex gangliosides. The presentation of several adhesion molecules in the BBB and the serum levels of MCP-1 were not significantly different between GM2/GD2−/− and wild-type mice. However, several complex gangliosides have been shown to be present in a human cerebromicrovascular endothelial cell line (Duvar et al. 2000). Complex gangliosides themselves may be involved as adhesion molecules in the process of infiltration of the activated lymphocytes into the CNS. In the present study, we demonstrated that the number of the pathogenic T cells within the CNS on day 9 of active immunization EAE was significantly less in GM2/GD2−/− mice in comparison with that in wild-type mice. This indicates that the lack of complex gangliosides in the BBB may delay the immigration of pathogenic T cells into the CNS. Thus, the complex gangliosides may be involved in the T cell–endothelial cell interaction in the pathogenetic process of EAE.

GM2/GD2−/− mice not only lack complex gangliosides, but are rich in simple gangliosides, such as GM3 and GD3 (Takamiya et al. 1996). GD3 strongly affected IL-15-dependent immune responses of murine microglia (Gomez-Nicola et al. 2006). GD3 has also been reported as a natural ligand for NKT cells (Wu et al. 2003). The delay of EAE onset in this type of knockout mouse may therefore be due to the increased content of GM3 and GD3. This possibility should be examined in future investigations.
Materials and methods

Mouse

Wild-type C57BL/6 mice were purchased from Clea Japan (Tokyo, Japan). GM2/GD2−/− were originally obtained from Dr. K. Furukawa and have been described previously. The mice have been backcrossed to the C57BL/6 background for more than five generations. Genotyping of GM2/GD2−/− mice was performed by polymerase chain reaction as described elsewhere. These mice were maintained under specific pathogen-free conditions. All mice for experiments were 8–12 weeks old.

Peptides

MOG35−55 (single-letter amino acid code; MEVGWYRSPFS-RVVHYLRNGK) was synthesized by Tore Research Institute (Tokyo, Japan). The peptides were >90% pure, as determined by HPLC.

Induction and assessment of EAE

Mice were injected subcutaneously in the flank, bilaterally, with 200 mL of inoculum containing 100 mg of MOG 35−55 and 0.5 mg of mycobacterium tuberculosis H37Ra (Difco Laboratories, Detroit, MI) in incomplete Freund’s adjuvant. Pertussis toxin (List Biological Laboratories Inc., Campbell, CA, 200 ng) was injected intravenously on day 0 and day 2 after immunization. For EAE induction in the adoptive transfer model, recipient mice were injected intravenously with encephalitogenic cells, prepared as described below, and 200 ng of pertussis toxin. Immunized mice were examined daily for signs of EAE in a blind fashion. For proliferation assays, mice were immunized with peptide/CFA as described above, but the mice were not treated with pertussis toxin. A single-cell suspension was prepared from the draining LNs on day 10 after immunization. Cells were cultured in DMEM medium (Gibco, Grand Islands, NY) supplemented with 5 × 10−5 M 2-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin and streptomycin, and 1% autologous mouse serum, and seeded onto 96-well flat-bottomed plates (1 × 106 cells/well). The cells were restimulated with peptide for 72 h at 37°C under a humidified air condition with 5% CO2. To measure cellular proliferation, [3H]-thymidine was added (1 mCi/well) and uptake of the radioisotope during the final 18 h of culture was counted with a beta-1205 counter (Pharmanics, Uppsala, Sweden). To evaluate proliferative responses of LN cells to the peptide, we determined the Δc.p.m. value for cells in each well by subtracting the background c.p.m. and used the mean of these values to represent each mouse.

Cytokine ELISA

In parallel, the LN cells from immunized mice were cultured with peptide concentrations of 0, 1, 10, and 100 µg/mL. Supernatants from the cultures were harvested 48 h postactivation and tested for the presence of various cytokines. The concentrations of IFN-γ, IL-2, IL-4, and IL-10 in the supernatants were measured by a sandwich ELISA according to the manufacturer’s guideline (BD Biosciences, San Jose, CA). Limits of detection for IFN-γ, IL-2, IL-4, and IL-10 were 195 pg/mL, 25 pg/mL, 12.5 pg/mL, and 50 pg/mL, respectively.

Pathological analysis

On day 35 after immunization, mice were sacrificed. Brains and spinal cords were harvested and fixed in 10% neutral buffered formalin. Paraffin sections were stained with hematoxylin and eosin (HE)–Luxol fast blue stain to assess inflammation and demyelination. The numbers of inflammatory foci in the meninges and parenchyma were counted for each sample by a blinded observer, as described previously (Miyamoto et al. 2005). For immunopathological analysis, the brain and spinal cord were obtained same as described above on day 7, 10, 14 after immunization of EAE. Thin-sliced (10 µm) frozen sections were fixed with acetone, and stained with HE, Luxol fast blue, and antibodies for adhesion molecules. ICAM-1 (CD54), VCAM-1 (CD106), E-selectin (CD62E), and P-selectin (CD62P) (BD Biosciences) were stained following the protocols provided by BD Bioscience.

The studies have been reviewed and approved by the local ethics committee in Kinki University.

Analysis of infiltrating cells isolated from CNS

Wild-type and GM2/GD2−/− mice were anesthetized with diethyl ether on day 9 after induction of EAE. After perfusion with PBS, brain and spinal cord were removed and homogenized. After washing with PBS, mononuclear cells were isolated using Percoll gradient (Amersham Biosciences, Piscataway, NJ) and were counted (Miyamoto et al. 2006). The cells were stained with a PE-labeled anti-CD4 antibody (BD Biosciences), and were analyzed by a flow-cytometer (BD FACS Calibur). Naïve mice were also analyzed the infiltrated cell in CNS as the same method.

Preparation of cells for EAE induction in the adoptive transfer model

To prepare MOG-specific cells that were able to induce EAE in the adoptive transfer model, mice were immunized with MOG/CFA in the same fashion as when inducing EAE, but no pertussis toxin was administered. Draining LNs were collected 10 days later, and a single-cell suspension was prepared. The cells were stimulated with 30 µg/mL MOG35−55 in 24-well flat-bottomed plates (5 × 106 cells/well) in the T-cell medium (RPMI media enriched with 10% FBS, 2 mM L-glutamine, 5 × 10−5 M 2-ME, nonessential amino acids, sodium pyruvate, and penicillin/streptomycin). Recombinant mouse IL-12 was added (100 U/mL) to the cultures for 72 h at 37°C under a humidified air condition with 5% CO2. To measure cellular proliferation, [3H]-thymidine was added (1 mCi/well) and uptake of the radioisotope during the final 18 h of culture was counted with a beta-1205 counter (Pharmanics, Uppsala, Sweden). To evaluate proliferative responses of LN cells to the peptide, we determined the Δc.p.m. value for cells in each well by subtracting the background c.p.m. and used the mean of these values to represent each mouse.

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In parallel, the LN cells from immunized mice were cultured with peptide concentrations of 0, 1, 10, and 100 µg/mL. Supernatants from the cultures were harvested 48 h postactivation and tested for the presence of various cytokines. The concentrations of IFN-γ, IL-2, IL-4, and IL-10 in the supernatants were measured by a sandwich ELISA according to the manufacturer’s guideline (BD Biosciences, San Jose, CA). Limits of detection for IFN-γ, IL-2, IL-4, and IL-10 were 195 pg/mL, 25 pg/mL, 12.5 pg/mL, and 50 pg/mL, respectively.

Pathological analysis

On day 35 after immunization, mice were sacrificed. Brains and spinal cords were harvested and fixed in 10% neutral buffered formalin. Paraffin sections were stained with hematoxylin and eosin (HE)–Luxol fast blue stain to assess inflammation and demyelination. The numbers of inflammatory foci in the meninges and parenchyma were counted for each sample by a blinded observer, as described previously (Miyamoto et al. 2005). For immunopathological analysis, the brain and spinal cord were obtained same as described above on day 7, 10, 14 after immunization of EAE. Thin-sliced (10 µm) frozen sections were fixed with acetone, and stained with HE, Luxol fast blue, and antibodies for adhesion molecules. ICAM-1 (CD54), VCAM-1 (CD106), E-selectin (CD62E), and P-selectin (CD62P) (BD Biosciences) were stained following the protocols provided by BD Bioscience.

The studies have been reviewed and approved by the local ethics committee in Kinki University.

Analysis of infiltrating cells isolated from CNS

Wild-type and GM2/GD2−/− mice were anesthetized with diethyl ether on day 9 after induction of EAE. After perfusion with PBS, brain and spinal cord were removed and homogenized. After washing with PBS, mononuclear cells were isolated using Percoll gradient (Amersham Biosciences, Piscataway, NJ) and were counted (Miyamoto et al. 2006). The cells were stained with a PE-labeled anti-CD4 antibody (BD Biosciences), and were analyzed by a flow-cytometer (BD FACS Calibur). Naïve mice were also analyzed the infiltrated cell in CNS as the same method.
Funding
This work was supported in part by grants-in-aid for Scientific Research (17046022) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by a Health Sciences Research Grant (Research on Psychiatric and Neurological Diseases and Mental Health) from the Ministry of Health, Labour and Welfare of Japan.

Conflict of interest statement
None declared.

Abbreviations
APC, antigen-presenting cell; BBB, blood–brain barrier; CNS, central nervous system; COX-2, cyclooxygenase-2; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; GM2/GD2−/−, GM2/GD2 synthase knockout mice; HE, hematoxylin and eosin; LN, lymph node; MOG, myelin-oligodendrocyte glycoprotein; MAG, myelin-associated glycoprotein; NO, nitric oxide; PNS, peripheral nerve system; TNF-α, tumor necrosis factor-alpha.

References


