clarify anti-AQP4 antibody status in these patients and compare differences in T cell reactivities to myelin proteins between anti-AQP4 antibody-positive and -negative conditions.

METHODS

Subjects and antigen-specific responses of established T cell lines

T cell lines were originally established from 11 patients with MS (three men and eight women) according to the McDonald criteria and seven healthy controls.[6] The median age was 48 years (range 28–64 years), while the median disease duration was 8.5 years (range 1–23 years). Briefly, T cell lines specific to the myelin self-peptides were established from peripheral blood mononuclear cells (PBMCs)[6], using 64 overlapping peptides of 16- to 21-amino acids in length, corresponding to the primary sequences of ^{196}MBP (amino acids 1–196), ^{276}PLP (amino acids 1–276), and ^{218}MOG (amino acids 1–218), including the exon 1–3 and exon 4–6 junctions of MBP.[6] Antigen-specific proliferation of the T cell lines was determined using peptide-pulsed PBMCs, as follows: The T cell lines ($3x10^4$) was cultured with irradiated (3,000 cGy) PBMCs for 72 h and pulsed with 1 μCi/well of [^{3}H] thymidine for the last 16 h. Test wells were considered to be positive with a stimulation index >2.0 and with a Δcpm (antigen-stimulated cpm minus non-stimulated cpm) >1,000 and at least three standard deviations above the mean cpm of unstimulated control wells. Blocking of the proliferative response was investigated by adding the following anti-HLA class II monoclonal antibodies (mAbs): Hu-4 (anti-HLA-DRB1+DRB5 monomorphic), 1a3 (anti-HLA-DQ monomorphic) and B7/21 (anti-HLA-DP monomorphic). The original T cell reactivity data, which have been previously described[6], were used for the present analyses.

Detection of anti-AQP4 antibody

Anti-AQP4 antibody was detected by an indirect immunofluorescence method using green fluorescent protein (GFP)-AQP4 fusion protein-transfected human embryonic kidney cells (HEK-293), as previously described.[5,7]

RESULTS

Anti-AQP4 antibody was detected in five of the eleven patients; these patients fulfilled the criteria for NMO[8] or NMO spectrum disorders.[9] Reactivities to MOG, PLP and MBP were detected in T cell lines established from 5/5, 3/5 and 3/5 of the anti-AQP4 antibody-positive patients with NMO/NMO spectrum disorders, respectively, and from 5/6, 4/6 and 4/6 of the anti-AQP4 antibody-negative patients with MS, respectively (Table 1). T cells reactive for myelin antigens from four of the five anti-AQP4 antibody-positive patients showed inter- or intra-molecular epitope spreading, while the same was true for five of the six anti-AQP4 antibody-negative MS patients (Figure 1).

DISCUSSION

Intramolecular epitope spreading is defined as a phenomenon in which T cells initially react only to a major or dominant antigenic epitope of an immunized antigen, and later show reactivity to other secondary or cryptic epitopes of the same immunogen. Intermolecular epitope spreading is defined as a phenomenon in which T cells initially reactive to only the immunized antigen molecule later demonstrate reactivity to other non-immunized molecules. Thus, reactivity of T cell lines to multiple sites of an antigenic molecule is regarded as intramolecular epitope spreading, while reactivity of T cell lines to multiple antigenic molecules is regarded as intermolecular epitope spreading. Inter- and intra-molecular epitope spreading of T cell lines is usually observed

Table 1: Proliferative responses to each myelin protein

Myelin protein	Anti-AQP4 antibody-positive NMO/NMO spectrum disorders	Anti-AQP4 antibody-negative MS	Healthy control
MBP	3/5	4/6	2/7
PLP	3/5	4/6	2/7
MOG	5/5	5/6	3/7

MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; PLP, proteolipid protein

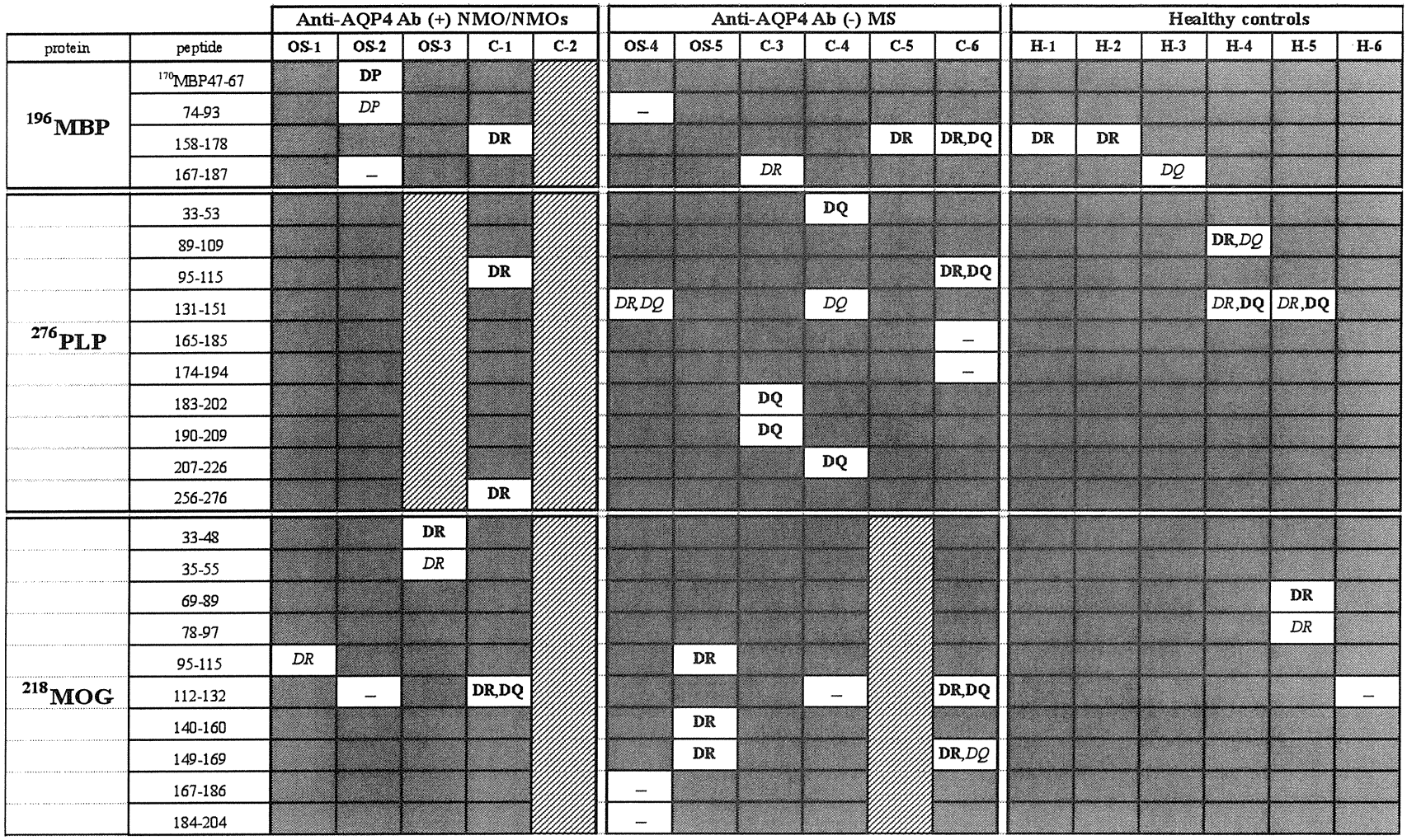

Figure 1. Myelin protein-reactive T cell lines characterized for peptide specificity and HLA restriction. T cell lines demonstrating either proliferative responses (open box) or no response (closed box) to peptides are shown. The hatched box indicates that the relevant epitope of T cell lines could not be determined because of a low response to the myelin peptide mixture. The restriction by HLA class II molecules of T cell lines is indicated in bold letters when anti-HLA class II mAb inhibited the proliferative response of T cell lines by ≥80%. Italic letters and (-) indicate that the mAb directed against the corresponding HLA class II molecules inhibited T cell proliferation by 50–80% and ≤50%, respectively.

Anti-AQP4 Ab: anti-aquaporin-4 antibody; HLA: human leukocyte antigen; mAb: monoclonal antibody; TCL: T cell lines; (+): positive; (-): negative.

in individuals whose T cells are stimulated or sensitized in vivo by specific antigen(s), whereas T cell lines stimulated or sensitized in vitro by antigen(s) during culture only react to neither multiple epitopes of the same antigen nor multiple antigens.

In the present study, T cell lines from healthy subjects also showed some reactivity to myelin proteins, but this was limited to one or two site(s) of a single myelin protein (five out of six cases demonstrated such a pattern). By contrast, T cell lines from four of five anti-AQP4 antibody-positive NMO/NMO spectrum disorders patients and five of six anti-AQP4 antigen-negative MS patients showed reactivity to multiple sites of multiple myelin proteins. It is therefore suggested that inter- and intra-molecular epitope spreading of T cell lines against myelin proteins occurs in most anti-AQP4 antibody-positive patients with NMO/NMO spectrum disorders and most anti-AQP4 antibody-negative ones with MS, but not in healthy subjects. The inter- and intra-molecular epitope spreading observed in anti-AQP4 antibody-positive patients indicates that T cells are already sensitized in vivo against major myelin proteins.

Recently, it was shown that sera from NMO patients with anti-AQP4 antibody can damage astrocytes in vivo following induction of experimental autoimmune encephalomyelitis by MBP-specific T cells.[10-12] It thus appears that encephalitogenic T cells are required for anti-AQP4 antibodies to exert their effects efficiently. Accordingly, the myelin protein-specific T cells found in anti-AQP4 antibody-positive NMO/NMO spectrum disorders patients may contribute to the initiation of CNS inflammation, and thereafter, anti-AQP4 antibody may invade the CNS and damage astrocytes in the presence of complement.

However, our T cell lines were established from the patients at certain periods after disease onset. Thus, myelin protein-specific T cell responses may have been secondarily developed after disease onset as a result of intermolecular epitope spreading. It will therefore be critical to study which CNS antigens T cells target at the time of initial attack, to elucidate the mechanisms underlying CNS anti-AQP4 autoimmunity. Nevertheless, it is possible that the myelin protein-specific T cells found in anti-AQP4 antibody-positive patients contribute to the development of inflammatory demyelination either primarily or secondarily.

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Research Paper

Multiple Sclerosis

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Altered production of brain-derived neurotrophic factor by peripheral blood immune cells in multiple sclerosis

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Abstract

Background: Within multiple sclerosis lesions, brain-derived neurotrophic factor is detected in neurons and immunocytes.

Objective: To clarify brain-derived neurotrophic factor production by peripheral blood immunocytes and its relationship with clinical parameters in multiple sclerosis.

Methods: Serum brain-derived neurotrophic factor levels were measured by conventional enzyme-linked immunosorbent assay while brain-derived neurotrophic factor production by immunocytes was determined by an *in situ* enzyme-linked immunosorbent assay in 74 multiple sclerosis patients, 32 healthy controls, and 86 patients with other neurological diseases. The tyrosine kinase receptor *TrkB* expression level in peripheral blood mononuclear cells was measured by real-time polymerase chain reaction.

Results: Multiple sclerosis patients showed significantly higher serum brain-derived neurotrophic factor levels than healthy controls and patients with other neurological diseases. Multiple sclerosis patients with high brain-derived neurotrophic factor levels were younger, and showed fewer relapse numbers than those with low brain-derived neurotrophic factor levels. Brain-derived neurotrophic factor production by T cells increased with age in healthy controls, but not in multiple sclerosis patients. Interferon beta induced a significant increase in serum brain-derived neurotrophic factor levels. Brain-derived neurotrophic factor production from T cells and *TrkB* expression levels in peripheral blood mononuclear cells were significantly enhanced in interferon beta-treated multiple sclerosis patients compared with untreated ones.

Conclusions: A high brain-derived neurotrophic factor level is related to early mild disease in young multiple sclerosis patients. Interferon beta potentiates brain-derived neurotrophic factor production and brain-derived neurotrophic factor receptor expression in peripheral blood mononuclear cells, which may act beneficially.

Keywords

Brain-derived neurotrophic factor, BDNF, multiple sclerosis, MS, interferon beta, IFNβ, T cells, age

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Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS). It is characterized by episodes of acute neurological dysfunction during the relapsing–remitting (RR) phase, leading to partial or full recovery.[1] However, with time, recovery from each episode becomes incomplete and persistent symptoms accumulate.[1] Thus, it is critical to understand the neuroprotection and repair mechanisms operating in this condition.

Among neuroprotective factors, brain-derived neurotrophic factor (BDNF), a member of the neurotrophin (NT) family which includes nerve growth factor (NGF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5),[2] has recently received a lot of attention in MS research. BDNF has been shown to promote neuronal survival after experimental axotomy[3] and to enhance oligodendrocyte proliferation and myelination.[4]

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Not only neurons but also immune cells, such as T cells, B cells, and monocytes, produce BDNF *in vitro* and in inflamed CNS lesions in MS patients.[5] Furthermore, tyrosine kinase receptor *TrkB*, a receptor for BDNF, was detected in reactive astrocytes within MS plaques and neurons in the immediate vicinity of such lesions.[6] Thus, BDNF and *TrkB* are supposed to be key players in neuroprotective immunity.[7]

There is accumulating evidence for a functional role of BDNF in the periphery, because BDNF is detected in both human serum and plasma.[8] More than 90% of blood BDNF is stored in platelets and can be released in serum on activation or clotting, which explains the 50–200-fold higher levels of BDNF in serum than in plasma.[8,9] Platelets and megakaryocytes have low levels of mRNA for BDNF;[8,10] however, platelets bind and internalize BDNF from other sources via the blood circulation, through high affinity receptors other than *TrkB*.[10] Alternative sources of blood BDNF identified to date include lymphocytes, monocytes,[5] eosinophils,[11] vascular endothelial cells,[12] and vascular smooth muscle.[13] Human platelets are known to circulate for about 10 days in peripheral blood,[14] while BDNF protein circulates in plasma for less than an hour.[15] Thus, serum or platelets are more stable components of blood to measure BDNF levels than plasma. Since BDNF is known to cross the blood–brain barrier in both directions, a substantial portion of circulating BDNF might originate from neurons and glia cells of the CNS.[16] Karege et al.[17] observed a positive correlation between serum and cortical levels of BDNF, indicating that cortical BDNF is a possible candidate source for circulating BDNF, and that peripheral measurement of BDNF could be used as a surrogate measure for BDNF levels in the CNS. Collectively, the presence of high levels of BDNF in platelets and serum suggests a role of circulating BDNF in neural repair at injured sites.

While no study reports platelet BDNF release in MS, there have been several studies describing circulating BDNF levels: serum BDNF levels are similar in relapsing–remitting MS (RRMS) patients in remission and controls;[18] they are lower in RRMS patients, either at relapse or in remission, than in healthy controls;[19] and they are higher in patients in relapse than in those in remission.[20] Concerning BDNF production by immune cells in MS, some studies have revealed that BDNF production by peripheral blood mononuclear cells (PBMCs) is higher during relapse and the recovery phase than during the stable phase in RRMS patients,[20,21] and that it is significantly associated with contrast-enhanced lesion volumes.[22] BDNF production by PBMCs was also reported to be higher in RRMS patients than in secondary progressive MS (SPMS) or primary–progressive MS (PPMS) patients.[20,23] In contrast, other studies have reported that BDNF production by PBMCs is lower in RRMS patients in remission than in controls.[24]

Regarding the effects of immunomodulatory therapies, PBMCs from glatiramer acetate (GA)-treated RRMS patients produced higher amounts of BDNF when compared with PBMCs from untreated RRMS patients and controls.[25] GA also significantly increased the serum levels of BDNF in MS patients,[19] especially in responders.[26] However, there is conflicting data on the effects of interferon beta (IFNβ) treatment on BDNF production; enhanced production of BDNF by PBMCs from IFNβ-treated patients[18] and by *in vitro* IFNβ-treated PBMCs[23] were reported in some studies, while no effect of IFNβ on BDNF expression was observed in others.[25,27] Thus, there is no consensus regarding serum BDNF levels and BDNF production by peripheral blood immune cells in MS patients, or their modification by IFNβ, and there have been no studies regarding BDNF levels and production in Asian MS patients in general. Therefore, in the present study, we aimed to measure serum BDNF levels, as well as BDNF production and *TrkB* expression by PBMCs, in Japanese MS patients with and without IFNβ treatment, and ascertain their relationships with clinical parameters. Our study is the first to measure BDNF production by separated T cells and monocytes from MS patients using an *in situ* enzyme-linked immunosorbent assay (ELISA), and the first to assess *TrkB* expression in PBMCs from untreated MS patients.

Methods

Subjects

In the present study, patients with MS, who were diagnosed as clinically definite MS according to the McDonald's criteria,[28] at the MS clinic in the Department of Neurology, Kyushu University Hospital between 1999 and 2008, were enrolled after informed consent was obtained. For conventional ELISA experiments to determine serum BDNF levels, 74 MS patients, 32 age-matched healthy controls (HCs), 29 patients with amyotrophic lateral sclerosis (ALS), 28 with spinocerebellar degeneration (SCD), and 29 with human T-cell lymphotropic virus associated myelopathy (HAM) were enrolled (Table 1). The disability status of the MS patients was scored by one of the authors (JK) throughout the study, according to the Kurtzke's Expanded Disability Status Scale (EDSS).[29] Among the MS patients, 66 patients had RRMS and eight had SPMS; no patients with PPMS were recruited. MS patients were clinically classified into two subtypes, opticospinal MS (OSMS) and conventional MS (CMS), as described previously.[30]

Table 1. Demographic features of subjects

	Number of patients (male/female)	Age at examination (years)	Disease duration (months)	EDSS	Number of relapses	Annualized relapse rates	RRMS/ SPMS	OSMS/ CMS	AQP4 (+)	NMO
Conventional ELISA for serum BDNF										
Untreated MS	74 (19/55)	39.9 ± 11.9	126.6 ± 115.4	3.38 ± 2.04	5.39 ± 4.88	0.94 ± 1.02	66/8	31/43	12	12
IFNβ-treated MS	12 (4/8)	40.8 ± 8.1	80.2 ± 51.1	2.79 ± 2.23	5.17 ± 6.07	1.97 ± 1.74	9/3	1/11	0	0
HCs	32 (15/17)	34.4 ± 10.2								
ALS	29 (13/16)	60.1 ± 12.4								
SCD	28 (14/14)	59.9 ± 14.0								
HAM	29 (10/19)	53.0 ± 13.2								
BDNF production from T cells and monocytes										
Untreated MS	17 (5/12)	50.8 ± 15.5	200.8 ± 122.9	3.26 ± 2.69	4.76 ± 3.73	0.31 ± 0.19	14/3	6/11	1	0
IFNβ-treated MS[a]	8 (3/5)	42.0 ± 8.3	91.9 ± 68.9	2.5 ± 2.2	3.13 ± 2.23	0.54 ± 0.38	6/2	0/8	0	0
HCs	18 (8/10)	44.9 ± 17.0								
TrkB expression in PBMCs										
Untreated MS	15 (1/14)	52.5 ± 15.8	220.7 ± 138	4.03 ± 2.45	6.87 ± 6.5	0.38 ± 0.27	13/2	4/11	0	0
IFNβ-treated MS[b]	11 (3/8)	37.2 ± 12.6	106 ± 68.7	2.64 ± 2.68	7.82 ± 8.22	0.79 ± 0.52	10/1	2/9	0	0
HCs	21 (10/21)	50.4 ± 15.2								

[a]Seven on IFNβ-1b and one on IFNβ-1a.
[b]Ten on IFNβ-1b and one on IFNβ-1a.
ALS, amyotrophic lateral sclerosis; AQP4 (+), anti-AQP4 antibody-positive; BDNF, brain-derived neurotrophic factor; CMS: conventional multiple sclerosis; EDSS, Expanded Disability Status Scale;[29] ELISA, enzyme-linked immunosorbent assay; HAM: human T-cell lymphotropic virus associated myelopathy; HCs, healthy controls; IFNβ, interferon beta; MS, multiple sclerosis; NMO: neuromyelitis optica; OSMS, opticospinal multiple sclerosis; PBMCs, peripheral blood mononuclear cells; RRMS, relapsing–remitting multiple sclerosis; SCD, spinocerebellar degeneration; SPMS, secondary progressive multiple sclerosis; *TrkB*, tyrosine kinase receptor.

There were 31 patients with OSMS (42%) and 43 patients with CMS (58%). The ages at examination were not significantly different between the two subtypes of patients (mean ± SD in years; 42.6 ± 14.3 in OSMS and 37.8 ± 9.3 in CMS). Anti-aquaporin-4 (AQP4) antibody was tested in all MS patients as previously described,[31,32] and only 12 were positive for the antibody (10 OSMS patients and two CMS patients); 62 were negative. Nine patients with anti-AQP4 antibody and three patients without anti-AQP4 antibody also fulfilled the criteria for neuromyelitis optica (NMO).[33] Thirty-one samples were obtained from 28 MS patients in the relapse phase, and 85 samples were obtained from 57 MS patients in the remission phase. All of the recruited patients were untreated for at least 6 months before study entry. Relapse was defined by the appearance of new neurological symptoms lasting at least 48 h in a patient who had been neurologically stable or improving for the previous 30 days, accompanied by objective changes on neurological examination. Thus, in the present study, the relapse phase was regarded as within 1 month after the onset of acute exacerbation, while the remission phase was regarded as either the stable stage or more than 1 month after exacerbation. We classified MS patients under treatment as clinical responders and suboptimal responders to IFNβ therapy, based on EDSS progression and the number of relapses; the occurrence of more than one relapse or an increase in EDSS score of at least 1 point during the one year of IFNβ treatment, according to Pozzilli et al.[34]

For the assay of BDNF production from T cells and monocytes using an *in situ* ELISA, 17 untreated MS patients, eight IFNβ-treated MS patients, and 18 HCs were enrolled (Table 1). For examination of *TrkB* expression in PBMCs, 15 untreated MS patients, 11 IFNβ-treated MS patients, and 21 HCs were enrolled (Table 1). The IFNβ-treated patients had received the drug for at least 6 months before the commencement of the study and none underwent any additional immunomodulatory therapy while they were on IFNβ.

Conventional ELISA for serum BDNF levels

Serum samples of MS patients and controls were stored at −80°C until use. Serum BDNF levels were determined using sandwich ELISA kits for BDNF (Promega, Madison, Wisconsin, USA) according to the procedure supplied by the manufacturer. In this procedure, flat-bottomed 96-well plates were coated with anti-human BDNF monoclonal antibody (mAB) to bind soluble BDNF, and the plates were incubated overnight at 4°C. After washing with wash buffer (Tris-HCl, pH 7.6), and blocking for nonspecific binding with Block & Sample

Buffer, the plates were incubated at room temperature for 1 h without shaking and washed once. Samples were diluted 80-fold with a calibrator prior to the assay. One hundred microliters of samples and BDNF standards, in duplicate, were added to the appropriate wells and the plates were incubated for 2 h at room temperature. A second BDNF-specific polyclonal antibody was added and the plates were incubated for 2 h at room temperature so that the captured BDNF could bind the polyclonal antibody. After washing, the amount of specifically bound polyclonal antibody was then detected using species-specific anti-IgY antibody conjugated to horseradish peroxidase (HRP) as a tertiary reactant. Unbound conjugate was removed by washing, which was followed by incubation with a chromogenic substrate and stopping the reaction with 1 N hydrochloric acid. The absorbencies were measured at 450 nm using an automatic ELISA microplate reader (IMMUNO-MINI NJ-2300, Tokyo, Japan). The sensitivity of the assay (expressed as the minimum amount of BDNF that could be detected) was 15.6 pg/ml, and the intra- and inter-assay coefficients of variation were 6.0% and 8.5%, respectively.

In situ ELISA for BDNF produced by peripheral blood immunocytes

PBMCs were isolated from venous blood containing 0.2% ethylenediamine tetraacetic acid (EDTA), diluted in a 1:1 ratio with saline, and subjected to density gradient centrifugation for 20 min at 2000 × g at 20°C, using lymphoprep tubes (Nycomed Pharma AS, Oslo, Norway). Dead cells were removed using the MACS Dead Cell Removal Kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Viable PBMCs were determined by trypan blue exclusion. T cells and monocytes were purified from PBMCs by negative immunoselection (Miltenyi Biotec). The purity of the isolated T cells and monocytes was >90% as determined by flow cytometry using anti-CD3 IgG and anti-CD14 IgG and fluorescein isothiocyanate (FITC)-conjugated antibodies. Isolated T cells and monocytes were washed twice and resuspended in X-VIVO 15 (Takara, Tokyo, Japan) at a concentration of 5×10^6 cells per milliliter. Then, 200 µl of purified peripheral blood T cells and monocytes (1×10^5 cells) was added to the UV-sterilized 96-well ELISA plates precoated with anti-BDNF mAB, which were incubated at 37°C in a 5% CO_2-humidified atmosphere for 48 h. Forty-six hours after the start of the assay, BDNF samples used to generate the standard curve were incubated in the same plate as the cells. At the end of the cell culture period, plates were extensively washed to remove all cells and cell debris, and the anti-BDNF polyclonal antibody was applied, followed by subsequent steps according to Promega's ELISA protocol. All experiments were performed in duplicate.

Real-time PCR for TrkB expression

TrkB mRNA expression in PBMCs from MS patients and HCs was examined by real-time PCR. First, approximately 5×10^6 to 1×10^7 PBMCs were isolated from venous blood containing 0.2% EDTA by density gradient centrifugation for 10 min at 2000 × g at 20°C on Ficoll-Paque. mRNA was extracted from total cellular RNA using a commercially available mRNA isolation kit (RNeasy Mini Kit, Qiagen GmbH, Hilden, Germany). Reverse transcription was performed using 750 ng of mRNA from each sample in a 20 µl reaction for 10 min at 65°C followed by 30 min at 55°C according to the manufacturer's instructions (Transcriptor First Strand cDNA Synthesis Kit; Roche Diagnostics GmbH, Mannheim, Germany). As a control, 750 ng of mRNA from each sample was treated according to the same protocol with the addition of distilled water instead of the reverse transcriptase. Real-time PCR was performed on the LightCycler® Instrument (Bio-Rad MiniOpticon™, Bio-Rad Laboratories, Hercules, California, USA) using the DNA-binding dye SYBR Green I (Takara SYBR Premix Ex Taq™ II, Takara, Kyoto, Japan). Specific primers for PCR were designed against glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (sense: 5′-GAGTCAACGGATTTGGTC GT-3′; antisense: 5′-TTGATTTTGGAGGGATCT CG-3′; expected product length: 238 bp), and *TrkB* (sense: 5′-CGAGATTGGAGCCTAACAGT-3′; antisense: 5′-CACCAGGATCAGTTCAGACA-3′: expected product length: 272 bp). After an initial denaturation step at 95°C for 5 s, the PCR reaction was performed with an annealing temperature of 55°C for 10 s, followed by an extension phase at 72°C for 15 s. At the end of each extension phase, fluorescence was observed at 72°C. The PCR reaction was completed after 45 cycles. Melting point analysis was performed by heating the amplicon from 50 to 95°C and revealed the characteristic melting point for each product. After cooling to 40°C, the product was extracted from the capillary. Ten microliters of each reaction was separated on a 1.5% agarose gel and visualized by staining with ethidium bromide.

Statistical analysis

Statistical analyses of numerical variables were initially performed using the Kruskal–Wallis *H*-test. When a significant difference was found, the Mann–Whitney *U*-test was used to determine the significance of differences between subgroups. *K*-means cluster analyses were used to identify subgroups among the MS patients according to serum BDNF levels. For comparisons among the subgroups, we performed logistic regression analyses. All analyses were performed using JMP

6.0.3 (SAS Institute, Cary, North Carolina, USA). Changes in serum BDNF levels were compared using the Wilcoxon signed rank test.

Results

Serum BDNF levels determined by conventional ELISA

MS patients showed significantly higher serum BDNF levels than HCs, and patients with ALS, SCD, and HAM ($p < 0.0001$ in all, Figure 1A). Serum BDNF levels tended to be higher in the relapse phase than in the remission phase ($p = 0.094$, Figure 1B) while they were not significantly different between males and females (Supplementary Figure 1A). Serum BDNF levels in OSMS patients were significantly higher than those in CMS patients ($p = 0.015$, Figure 1C), while they were not significantly different between anti-AQP4 antibody-positive and antibody-negative patients, or between those who fulfilled the NMO criteria and those who did not (Supplementary

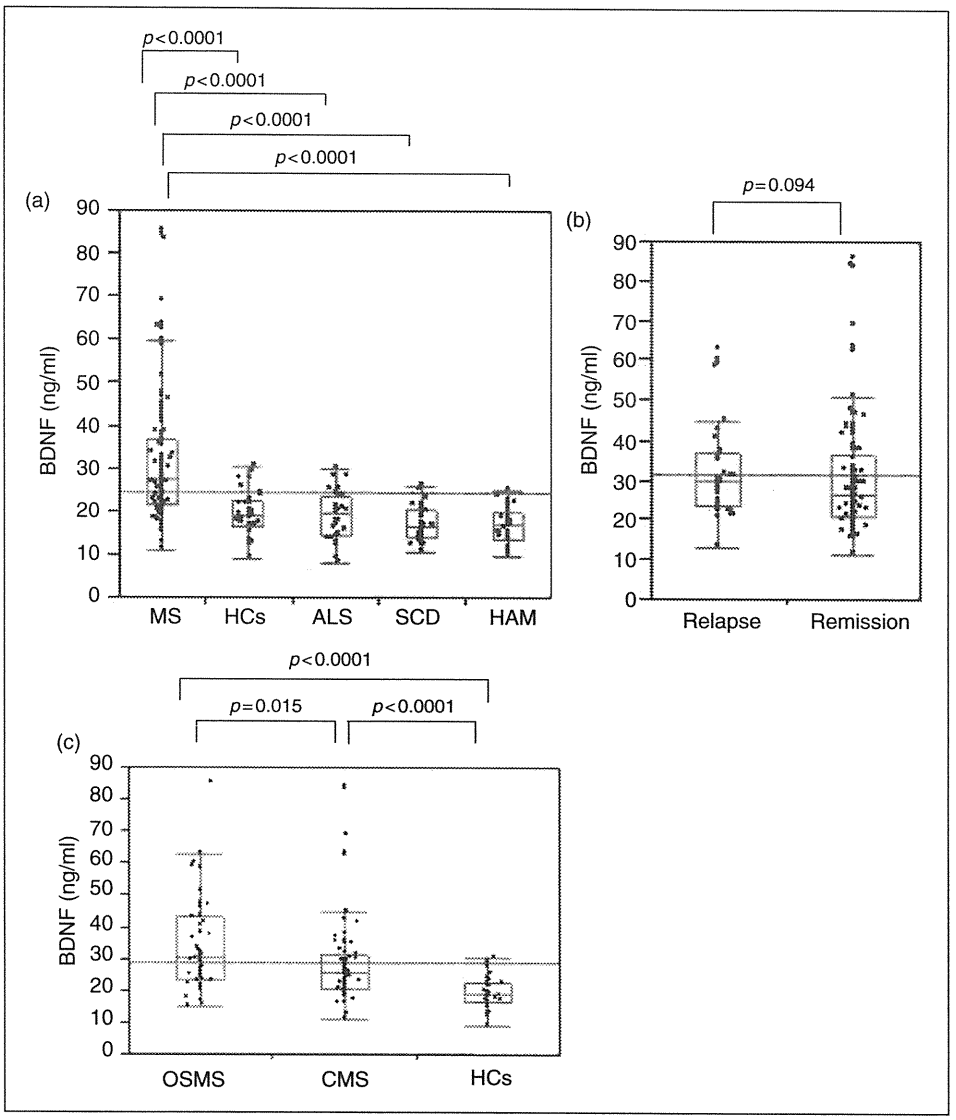

Figure 1. Serum BDNF levels determined by conventional ELISA. MS patients show significantly higher serum BDNF levels compared with HCs, and patients with ALS, SCD, and HAM (A). Serum BDNF levels tends to be higher in the relapse phase than in the remission phase (B). Serum BDNF levels are significantly higher in OSMS patients than in CMS patients, although both OSMS and CMS patients show significantly increased levels of BDNF compared with HCs (C).
ALS, amyotrophic lateral sclerosis; BDNF, brain-derived neurotrophic factor; CMS, conventional multiple sclerosis; ELISA, enzyme-linked immunosorbent assay; HAM, human T-cell lymphotropic virus associated myelopathy; HCs, healthy controls; MS, multiple sclerosis; *NS*, not significant; OSMS, opticospinal multiple sclerosis; SCD, spinocerebellar degeneration.

Figure 1B, C). Exclusion of anti-AQP4 antibody-positive patients and those who met the NMO criteria gave essentially the same results. Serum BDNF levels were not significantly different between RRMS (mean ± SD = 32 ± 14.9) and SPMS patients (mean ± SD = 24.9 ± 4.9), in part due to the small sample size of SPMS patients, while none of the SPMS patients had BDNF levels higher than 1 SD above the mean level in RRMS patients (Supplementary Figure 1D).

Correlation of serum BDNF levels with clinical parameters

Serum BDNF level showed a significant positive correlation with age at examination in HCs ($p = 0.042$,

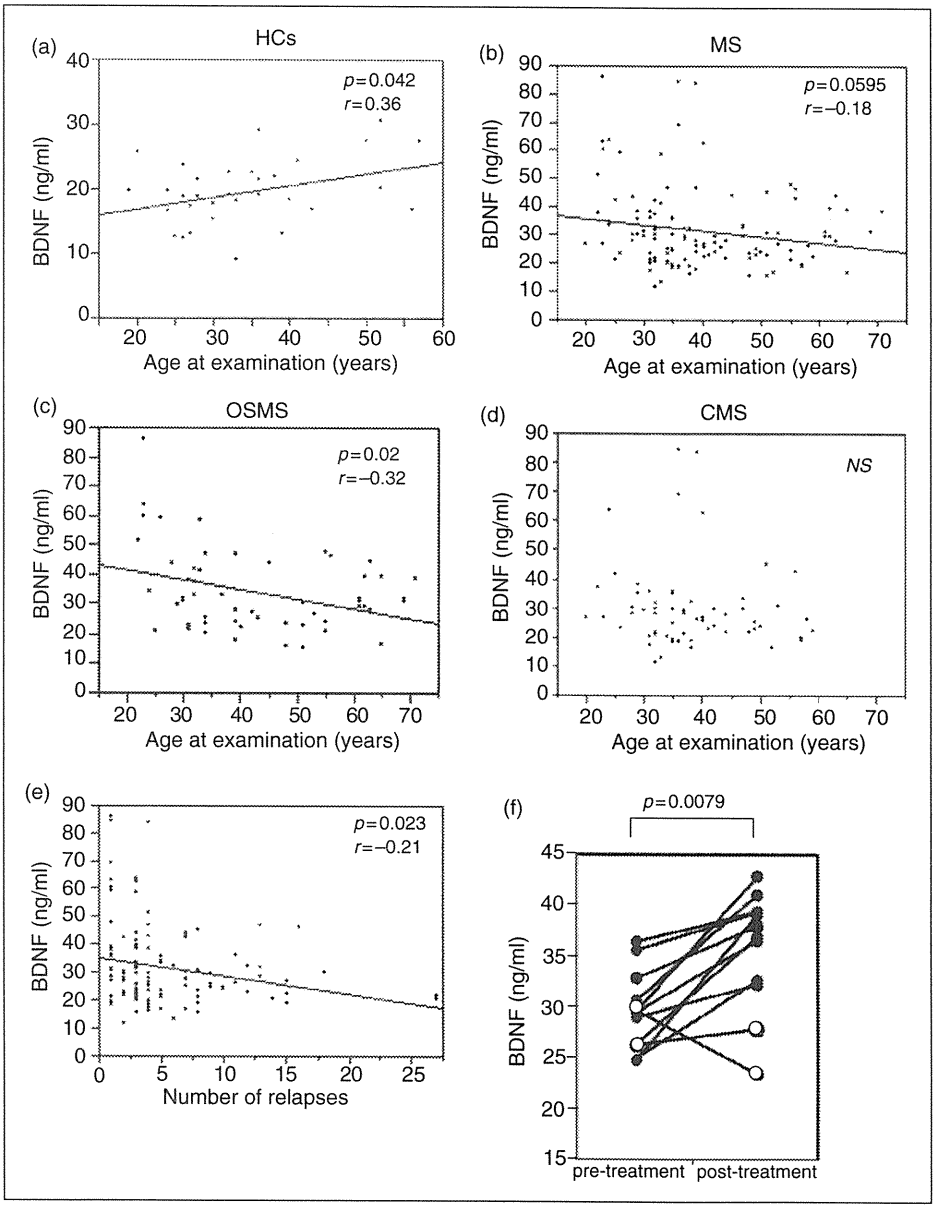

Figure 2. Relationship between serum BDNF levels and clinical parameters. Serum BDNF levels show a significant positive correlation with age at examination in HCs (A), whereas it tends to decrease with age in MS patients (B). Serum BDNF levels are significantly negatively correlated with age at examination in OSMS patients (C), but not in CMS patients (D). Serum BDNF level is also negatively correlated with the number of relapses (E). Serum BDNF levels are significantly higher after IFNβ treatment than before treatment (F). Closed circles represent responders, while open circles indicate suboptimal responders to IFNβ.
BDNF, brain-derived neurotrophic factor; CMS, conventional multiple sclerosis; HCs, healthy controls; MS, multiple sclerosis; *NS*, not significant; OSMS, opticospinal multiple sclerosis.

$r = 0.36$), while in MS patients it tended to show a weak negative correlation ($p = 0.059$, $r = -0.18$) (Figure 2A, B). Serum BDNF levels were significantly negatively correlated with age at examination in patients with OSMS ($p = 0.02$, $r = -0.32$), but not in those with CMS (Figure 2C, D). Serum BDNF level was negatively correlated with number of relapses ($p = 0.023$, $r = -0.21$) (Figure 2E) but not with disease duration, EDSS scores, or annualized relapse rate (Supplementary Figure 2A–C). There was no significant correlation between serum BDNF levels and EDSS after correcting for age and disease duration by multiple logistic regression analyses (data not shown). In addition, there was no significant correlation of serum BDNF levels at relapse (31 samples) with EDSS scores either at the peak of relapse or at three months after the relapse (Supplementary Figure 2D, E).

K-means cluster analyses revealed three subgroups on the basis of serum BDNF levels: high (58.1–85.2 ng/ml, $n = 10$), medium (31.4–50.8 ng/ml, $n = 30$), and low (11.2–31.0 ng/ml, $n = 76$). Logistic regression analyses of the subgroups identified by K-means clustering disclosed that as compared with the BDNF-low and -medium groups, the BDNF-high group showed significantly younger age at examination ($p = 0.0108$ and $p = 0.0237$, respectively) and fewer relapses ($p = 0.0123$ and $p = 0.0184$, respectively) (Supplementary Figure 3A, B). Disease duration had a tendency to be shorter in the BDNF-high group than in the BDNF-low and -medium groups ($p = 0.0644$ and

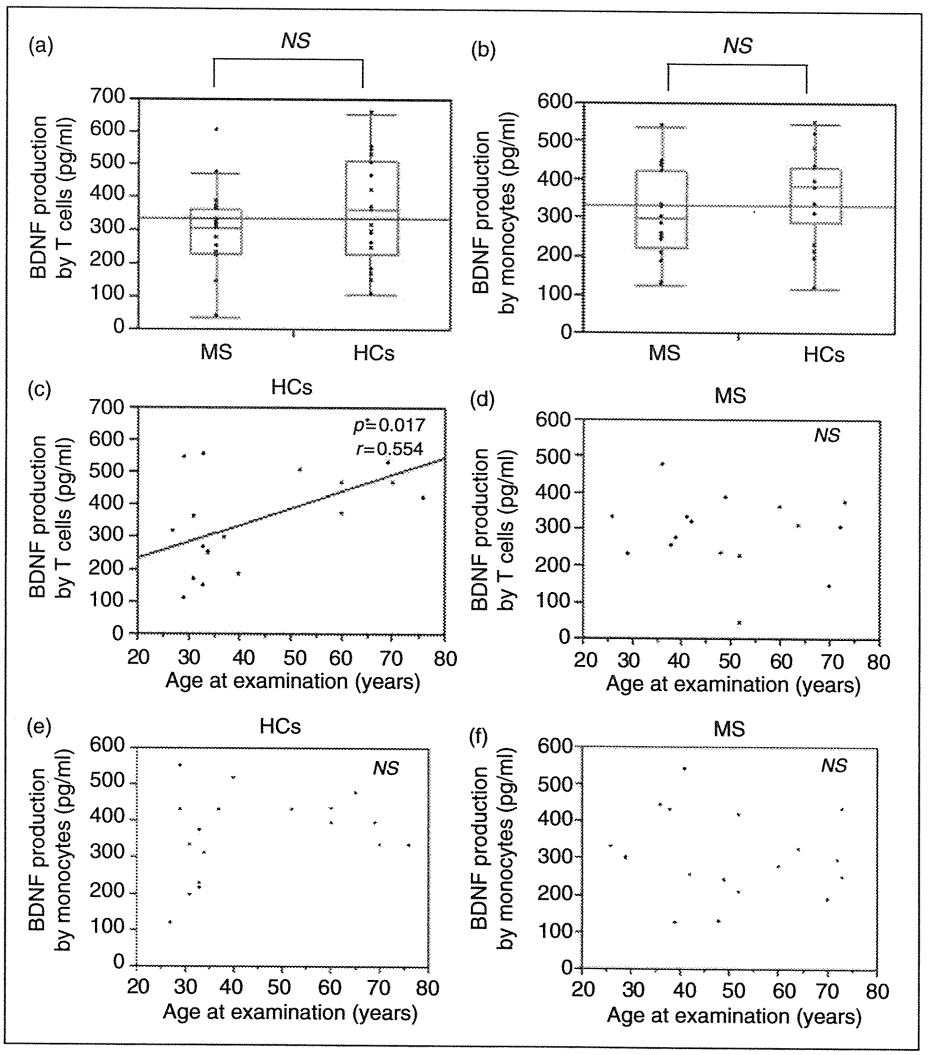

Figure 3. ELISA *in situ* analysis of BDNF production by immunocytes. BDNF production by T cells (A) and monocytes (B) is not significantly different between untreated MS patients and HCs. In HCs, BDNF production by T cells shows a significant positive correlation with age at examination (C). In MS patients, such a correlation between BDNF production and age is not seen (D). BDNF production by monocytes has no correlation with age in either HCs (E) or MS patients (F).
BDNF, brain-derived neurotrophic factor; ELISA, enzyme-linked immunosorbent assay; HCs, healthy controls; MS, multiple sclerosis; *NS*, not significant.

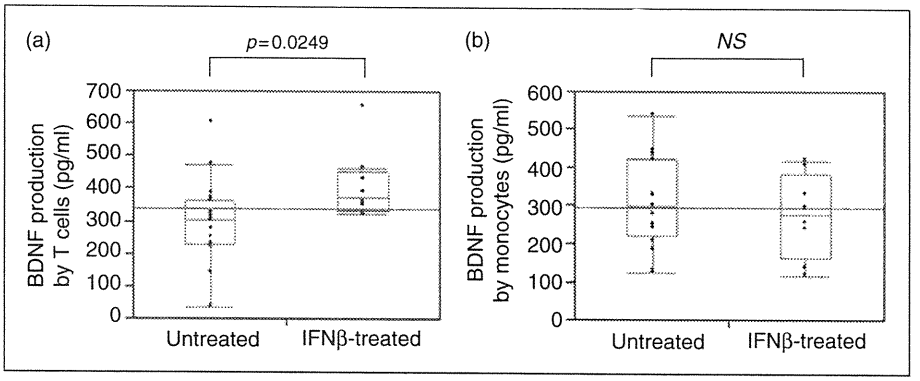

Figure 4. Comparison of BDNF production by immunocytes between IFNβ-treated MS patients and untreated ones. BDNF production by T cells shows a significant increase in IFNβ-treated MS patients compared with untreated ones (A). On the other hand, BDNF production by monocytes is not significantly different between IFNβ-treated and untreated patients (B).
BDNF, brain-derived neurotrophic factor; IFNβ, interferon beta; MS, multiple sclerosis; *NS*: not significant.

$p = 0.0994$, respectively), while neither EDSS scores nor annualized relapse rate differed significantly among the three groups (Supplementary Figure 3C–E). Introduction of IFNβ induced a significant increase in serum BDNF levels in MS patients ($p = 0.0079$) (Figure 2F).

Among the 12 IFNβ-treated patients, 10 were regarded as responders (nine were CMS patients and one was an OSMS patient who neither had anti-AQP4 antibody nor met the diagnostic criteria for NMO)[33] and two were regarded as suboptimal responders (both with CMS). The two suboptimal responders (both with CMS) had the lowest and the second lowest serum BDNF levels among the IFNβ-treated MS patients after IFNβ administration. The only patient who showed a decrease in serum BDNF levels after therapy was one of the two suboptimal responders, who had the lowest serum BDNF level after IFNβ administration.

BDNF production from peripheral blood immunocytes by in situ ELISA

Levels of BDNF production by T cells and monocytes were not significantly different between untreated MS patients and HCs (Figure 3A, B). In HCs, BDNF production by T cells demonstrated a significant positive correlation with age at examination ($p = 0.017$, $r = 0.554$), whereas in MS patients, such a correlation was not seen (Figure 3C, D). By contrast, BDNF production by monocytes had no correlation with age in either MS patients or HCs (Figure 3E, F). BDNF production by either T cells or monocytes was not correlated with disease duration, number of relapses, annualized relapse rate, or EDSS scores (data not shown). Levels of BDNF production by T cells and monocytes were not significantly different between RRMS and SPMS patients (data not shown). BDNF production by T cells was significantly increased in IFNβ-treated MS patients compared with untreated ones ($p = 0.0249$), while BDNF production by monocytes did not differ significantly with IFNβ treatment (Figure 4A, B). Among IFNβ-treated MS patients, six responders and two suboptimal responders showed similar levels of BDNF production by either T cells or monocytes (data not shown).

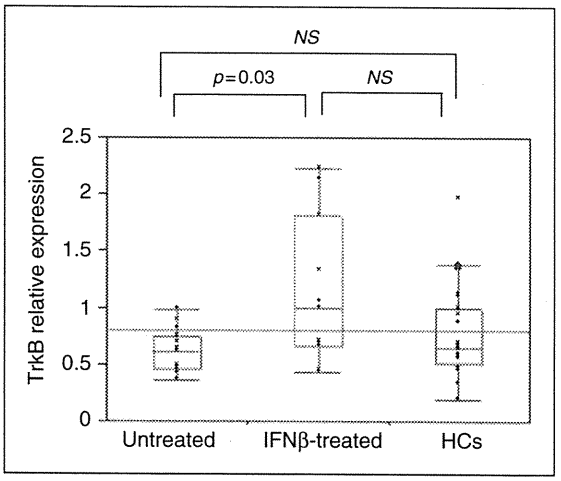

Figure 5. Real-time PCR analysis of *TrkB* expression levels. *TrkB* expression levels in PBMCs are significantly higher in IFNβ-treated MS patients than untreated ones, but are not significantly different either between untreated MS patients and HCs or between IFNβ-treated MS patients and HCs.
BDNF, brain-derived neurotrophic factor; HCs, healthy controls; IFNβ, interferon beta; MS, multiple sclerosis; *NS*, not significant.

Real-time PCR analysis of TrkB expression levels

TrkB expression levels in PBMCs were significantly higher in IFNβ-treated MS patients than in untreated ones ($p = 0.03$), but were not significantly different either between untreated MS patients and HCs or between

IFNβ-treated MS patients and HCs (Figure 5). *TrkB* expression levels were not correlated with age, disease duration, number of relapses, annualized relapse rate, or EDSS scores (data not shown). *TrkB* expression levels were not significantly different either between RRMS and SPMS patients or between the eight responders and the three suboptimal responders (data not shown).

Discussion

In the present study, we found that, compared with controls, BDNF levels in the sera of RRMS patients were significantly higher, especially in younger patients with fewer relapses and mild disease. Interestingly, a significant positive correlation of BDNF levels with age was found in HCs, whereas a trend toward a negative correlation was observed in MS patients. Such a reverse trend was apparent in OSMS patients while it was not in CMS patients. However, even the lower levels in older MS patients were compatible with those in HCs at the same ages. Thus, it is suggested that serum BDNF levels are increased in younger MS patients with an early disease course, but decreased in older patients in the late stage of the disease to the levels seen in HCs of similar ages. Thus, the reverse trend of BDNF levels in relation to age appears to be explained by the existence of young MS patients with high BDNF levels, but without anti-AQP4 antibody.

Our results are in line with previous studies showing that the PBMCs of RRMS patients produce more BDNF than those of SPMS patients.[20,23] Furthermore, BDNF levels in our series showed a tendency to be higher at relapse than in remission, which is in accordance with previous results demonstrating that BDNF production by PBMCs is higher in the active phase than in the stable phase in RRMS patients.[20,21] Therefore, at least in young patients, acute inflammation appears to induce enhanced BDNF production in peripheral blood during early relapses in MS patients, which is consistent with the concept of neuroprotective immunity.[7] Because BDNF production appears to decline during later relapses, such neuroprotective immunity may contribute to good recovery and tissue repair only in the early course of the disease. However, our results are somewhat discrepant from those studies reporting similar or lower serum BDNF levels in MS patients compared with controls.[18,19] Differences in the proportion of younger patients or ethnic backgrounds may account for these discrepancies. In the present study, considerable numbers of our MS patients presented with an OSMS phenotype, and most of these were seronegative for anti-AQP4 antibody (68% were seronegative), as reported previously.[31,32] Moreover, OSMS patients had significantly greater serum BDNF levels than CMS patients. It is the OSMS patients who show a significant negative correlation of BDNF level with age. Thus, young OSMS patients without anti-AQP4 antibody in the early course of the disease appear to have high BDNF levels, which may relate to the rare occurrence of a progressive course reflecting neuroaxonal degeneration in this subgroup.[35,36]

We could not find any increase in BDNF production by peripheral blood T cells or monocytes by an *in situ* ELISA, whereas previous studies have shown increased BDNF production by PBMCs.[21,27] Differences in assay methods, stimuli employed, and the number of patients studied may in part explain these discrepancies. Hamamcioglu and Reder[23] reported a decrease in BDNF production by unstimulated PBMCs in SPMS patients compared with HCs. Petereit et al.[27] described an increase in BDNF production by phorbol myristate acetate (PMA)- and ionomycin-stimulated PBMCs in RRMS patients in remission compared with HCs. Caggiula et al.[21] disclosed that BDNF production by unstimulated PBMCs in RRMS patients was higher in relapse than in remission. Lalive et al.[18] revealed that BDNF levels in PBMC lysates were higher in lysates of PBMCs from IFNβ-treated RRMS patients than in those from untreated ones. All of the above-mentioned studies were done with unseparated PBMCs using an ELISA of the culture supernatants or cell lysates, whereas we separated PBMCs into T cells and monocytes and employed an *in situ* ELISA. The only other study using separated T cells and monocytes, performed by Azoulay et al,[24] examined only two MS patients, and found similar BDNF levels to those in controls, which is in accord with our results.

In our hands, BDNF production by only T cells significantly increased with age in HCs, which may relate to an age-associated increase in serum BDNF levels in HCs. However, such a trend was completely absent in MS patients. Therefore, BDNF production by T cells in aged MS patients may be dampened, and this may partly account for the age-associated decrease in serum BDNF levels in MS patients. It is also possible that BDNF production may be up-regulated in other cell populations, such as B cells, platelets, and eosinophils, which are also known to produce it.[11,37,38] Further studies on these populations are required to clarify the source of elevated BDNF levels in sera from young MS patients. Alternatively, secreted BDNF may not be utilized efficiently in the periphery. However, because *TrkB* expression levels in PBMCs from untreated MS patients did not differ significantly from those in PBMCs obtained from HCs, a comparison that had not previously been made, this possibility seems less likely.

Several reports have indicated that serum BDNF levels are lower in drug-free subjects suffering from major depression than in healthy controls.[39–42] In our

series, no MS patient had major depression at the time of examination. However, follow-up studies may be necessary to clarify whether low BDNF patients experience future development of depression.

Finally, for the first time, we have shown that IFNβ treatment in MS patients potentiates BDNF production in T cells but not in monocytes, which may account for the increase in serum BDNF levels after introduction of IFNβ observed in the present study. These findings are in line with the results of previous studies showing that BDNF production by PBMCs from IFNβ-treated patients is enhanced,[18] and that GA, another disease-modifying drug for MS, also potentiates BDNF production in T cells.[43] We also found that *TrkB* expression levels in PBMCs were significantly elevated in IFNβ-treated MS patients compared with untreated ones. Among PBMCs, *TrkB* expression has been reported in human T[44,45] and B cells.[46] Although there is a need for further study to exactly identify the cell populations showing an enhancement of *TrkB* expression by IFNβ, IFNβ-induced enhanced BDNF production in T cells and increased levels of serum BDNF thus appear to at least partly contribute to its beneficial effects in MS. Therefore, enhancement of neuroprotective immunity, as seen in early MS patients, may be an important target for future disease-modifying therapy in MS.

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Conflict of interest statement

None declared.

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Neuromyelitis optica and opticospinal multiple sclerosis: Mechanisms and pathogenesis

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Abstract

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS) while neuromyelitis optica (NMO) is an inflammatory disease of the CNS that selectively affects the optic nerves and spinal cord. In Asians, MS is rare; however, when it appears, the selective and severe involvement of the optic nerves and spinal cord is characteristic. This form, termed opticospinal MS (OSMS), has similar features to the relapsing form of NMO in Western populations. Recently, a specific IgG against NMO, designated NMO-IgG, was discovered, and the relevant antigen was found to be aquaporin-4 (AQP4), one of the major water channel proteins in the CNS. Because NMO-IgG has been reported to be present in 30–60% of OSMS patients, OSMS in Asians has been suggested to be the same entity as NMO.

The sensitivity of NMO-IgG/anti-AQP4 antibody for NMO varies from 30% to 80%, while the specificity is 90–100%. Pathological studies on NMO have revealed perivascular immune complex (IgM, IgG and C9neo) deposition and extensive loss of AQP4 in active lesions, where myelin basic protein (MBP) staining was relatively preserved. IgG from NMO-IgG-seropositive NMO patients induces astrocyte death in culture in the presence of complements, and reproduces astrocyte loss *in vivo* when MBP-specific T cells are co-transferred to cause experimental autoimmune encephalomyelitis. It is thus postulated that the complement-activating anti-AQP4 antibody plays a pivotal role in the development of NMO lesions through astrocyte necrosis, and that demyelination is a secondary event.

However, in autopsied cases of NMO, we and others found that some demonstrated selective AQP4 loss while others showed preservation of AQP4, even in the acute lesions. We also found that, in some MS lesions, AQP4 was lost extensively far beyond the areas of myelin loss. In the CSF, proinflammatory cytokines such as IL-17, IL-8, IFNγ, and G-CSF are markedly elevated in OSMS patients, irrespective of the presence or absence of anti-AQP4 antibody. In OSMS and NMO patients, T cells reactive to myelin proteins show intra- and inter-molecular epitope spreading, suggesting that T cells are already stimulated with myelin antigens *in vivo*. These findings suggest that mechanism of NMO and OSMS in Asians is heterogeneous, anti-AQP4 antibody-related and -unrelated, and that not only anti-AQP4 antibody but also myelin-autoreactive Th17 or Th1 cells may also play a role in triggering CNS inflammation. Possible mechanisms for NMO and OSMS are discussed in this review.

Keywords: Multiple sclerosis; Neuromyelitis optica; Aquaporin-4; Opticospinal multiple sclerosis; Th17; Astrocyte necrosis; Asians and Westerners

1. Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) thought to be caused by autoimmune attacks targeting CNS myelin. The pathological hallmark in MS is sharply demarcated demyelinating plaques with axons relatively preserved. By contrast, neuromyelitis optica (NMO) is an inflammatory disease of the CNS selectively affecting the optic nerves and spinal cord. In this condition, longitudinally extensive spinal cord lesions (LESCLs) extending over three vertebral segments are said to be characteristic on magnetic resonance imaging (MRI). Pathologically, both axons and myelin are involved, resulting in necrotic cavitation. In Asians, MS is rare; however, when it appears, the selective but severe involvement of the optic nerves and spinal cord is characteristic [1]. This form, termed opticospinal MS (OSMS), has similar features to the relapsing form of NMO in Western populations [2]. The nosological position of NMO has long been a matter of

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debate. The recent discovery of a specific IgG against NMO, designated NMO-IgG, suggests that NMO is a distinct disease entity with a fundamentally different etiology from MS [3,4]. Because NMO-IgG has been reported to be present in about 50–60% of OSMS patients [3,5], OSMS in Asians has been suggested to be the same entity as NMO. However, the observations that NMO-IgG is not found in all cases of NMO or OSMS [5–7], and that 5–10% of classical MS patients also carry the antibody [3,5,7] cast doubt on the simple dichotomy of categorizing human demyelinating disease into MS and NMO. In Asians, the mechanism underlying the formation of LESCLs is heterogeneous, and the disease condition in those with NMO-IgG does not completely overlap with OSMS in Asians [6,7]. In this review, possible mechanisms underlying NMO with NMO-IgG and OSMS without the antibody are discussed.

2. History and nosological problems of neuromyelitis optica

The nosological position of NMO has been a matter of debate since Dević first summarized cases with optic neuritis and spinal cord disease [8]. Originally, NMO was considered to be a monophasic disease that simultaneously affected both the optic nerves and spinal cord. However, several reported cases showed a relapsing course [8–11] and, later, Wingerchuk et al. [2] proposed a nosological entity of relapsing NMO and described its diagnostic criteria. Some cases of relapsing NMO even showed general and local cerebral symptoms, such as Jacksonian seizure, headache, vomiting and dysarthria [8–11]. As well, pathologically, small foci of demyelinating plaques are frequently observed in postmortem brains from NMO patients [10,11]. The occurrence of relapse and brain symptoms, and the pathologically demonstrated presence of additional demyelinating plaques, indicate the existence of considerable overlap between MS and NMO. This situation imposes difficulty for differentiating NMO from MS clinically and pathologically.

3. History and features of MS in Asians

Before the late 1950s, MS was rarely reported in Asia countries. In 1958, Okinaka et al. [12] reported the clinical features of 270 cases of MS and allied disorders that had been diagnosed between 1890 and 1955. In this series, 65% had NMO, 24% had MS and 2% had Schilder's disease, while the other cases had unclassifiable diseases. Among the NMO cases, 48% showed a relapsing course and the authors found many intermediate cases between MS and NMO. Thereafter, in Japan and the rest of Asia, NMO has been used to describe monophasic cases showing bilateral optic neuritis and transverse myelitis within an interval of less than several weeks, and relapsing cases have usually been classified as MS.

A comparative study of MS between Japanese and British patients done by Shibasaki et al. [13] disclosed the characteristic features of MS in Asians. These included selective and severe involvement of the optic nerves and spinal cord, rapid progression, infrequent secondary progression, rare familial occurrence, and no association of MS patients as a group with any HLA allele. MS in Asians was thus considered to be modified from that seen in Western populations as a whole.

In 1996, Kira et al. [14] first reported different features between opticospinal (OSMS, Asian type MS) and conventional MS (CMS, Western type MS) and proposed clinical classification criteria for OSMS, namely, selective involvement of the optic nerves and spinal cord by clinical symptomatology with or without minor brainstem signs. Thereafter, phenotypic classification and characterization have been actively undertaken by Japanese researchers. These studies have revealed that 15–40% of MS cases in Japan are of an OSMS phenotype and have clarified the demographic features of OSMS [1]. Compared with CMS, OSMS has the following characteristic features in Asians: (1) higher age at onset; (2) female preponderance; (3) frequent relapses; (4) greater disability due to severe optic nerve and spinal cord damage; (5) fewer brain MRI lesions; (6) LESCLs extending over many vertebral segments on spinal cord MRI; (7) marked pleocytosis and neutrophilia in cerebrospinal fluid (CSF); and (8) absence of oligoclonal bands (OB) in CSF. Moreover, HLA association is also distinct between the two subtypes: *HLA-DRB1*1501* is associated with the CMS phenotype [14], as seen in Caucasian patients with MS, while *HLA-DPB1*0501* is associated with OSMS in Japanese [15]. On the other hand, CMS patients show similar features to MS in Westerners, including the same *HLA-DRB1*1501* association [1]. These observations suggest that the two subtypes have distinct mechanisms; however, there remains considerable overlap between the two disease entities, primarily because of the arbitrariness and ambiguity encompassed by the clinical findings-based classifications.

4. Discovery of NMO-IgG and its relevant antigen in NMO

NMO-IgG was originally found in 73% of NMO patients without brain lesions on MRI by immunohistochemical staining of mouse cerebellar tissue sections [3]. Later, the relevant antigen recognized by the antibodies was found to be aquaporin-4 (AQP4) [4]. Thereafter, AQP4- or GFP–AQP4 fusion protein-transfected cells have been used for immunostaining. AQP4 is one of the major water channel proteins in the CNS and is abundantly expressed throughout the CNS including the cerebrum and cerebellum. Although NMO mainly affects the optic nerves and spinal cord, Pittock et al. [16] reported that asymptomatic brain lesions on MRI are common in NMO patients with anti-AQP4 antibody. According to the revised criteria for NMO (Table 1), even the presence of symptomatic brain lesions does not exclude a

Table 1
The revised criteria for neuromyelitis optica.

Definite NMO
1. Optic neuritis
2. Acute myelitis
3. At least 2 of 3 supportive criteria
1. LESCL
2. Brain MRI not meeting Dx. criteria for MS
3. NMO-IgG (+)

Wingerchuk et al. (2006) (Ref. [17]).

diagnosis of NMO based on the presence of NMO-IgG [17]. According to Pittock et al. [18], brain lesions in NMO are preferentially observed in regions where AQP4 is abundantly present, such as the bilateral diencephalic regions adjacent to the third ventricles, the pontine tegmentum and cerebellum surrounding the fourth ventricles, and the periventricular white matter adjacent to the lateral ventricles.

5. Positivity rates of NMO-IgG and anti-aquaporin-4 antibody in various ethnic groups

5.1. Seroprevalence and sensitivity

One of the confounding problems concerning the involvement of NMO-IgG in the diagnosis of NMO is that NMO-IgG is not detected in all NMO patients (Table 2). In Caucasians, 73% were positive in Lennon's original report [3] and similar figures have also been reported elsewhere: 22/36 (61.1%) in Jarius et al. [19] and 21/37 (56.72%) in Paul et al. [20] were positive. Recently, Fazio et al. [21] conducted immunofluorescence, flow cytometry and radioimmunoprecipitation assays in Italian patients with NMO and found 30–47% were positive. In Africans and their descendants, much lower positivity rates were reported: 33.3% in Caribbean patients with NMO [22] and 5% in African–American patients with OSMS [23].

In Japanese, Nakashima et al. [5] reported detection of NMO-IgG in 63% of OSMS patients and 15% of CMS patients. Recently, the same group also reported that 20 of 22 NMO patients had the anti-AQP4 antibody, while none of the 53 MS patients did (90% versus 0%) [24]. In their series, all 22 NMO patients, all female, were defined as cases fulfilling all items of the 2006 NMO criteria [17] except for NMO-IgG-seropositive status. From reports before the discovery of NMO-IgG, the male to female ratio in relapsing NMO was 1:5 at most and 1:1 in the monophasic type [2]. Therefore, considering the extremely high female ratio in Takahashi's series [24], there appears to have been an obvious subject bias in their study. Tanaka et al. [25], in their selected series of MS patients, independently reported that anti-AQP4 antibody positivity rate was 16/26 (61.5%) in OSMS patients with LESCLs and 0/21 (0%) in CMS patients without LESCLs.

Table 2
Positivity rates for NMO-IgG/anti-AQP4 antibody among races.

Race	Disease	NMO-IgG/anti-AQP4 antibody (%)
Caucasians [3,19–21]	NMO	30–73*
Northern Japanese [5]	OSMS	63*
Southern Japanese [6]	OSMS	27*
Caribbean [22]	NMO	33
Indian [29]	NMO spectrum disorder	5*
African–American [23]	OSMS	5

* Measured by Mayo Clinic.

It is critical that anti-AQP4 antibody be examined in a blind fashion in a large number of consecutive MS patients covering the whole spectrum of MS and that the positivity rate be compared with that in NMO-IgG patients. We undertook such a study using serum samples with NMO-IgG status predetermined at the Mayo Clinic; we found that their anti-AQP4 antibody assay was 83.3% sensitive and 100% specific for NMO-IgG [6]. According to the results using this assay system, the anti-AQP4 antibody was positive in 27.1% (13/48) of OSMS patients, 5.6% (3/54) of CMS patients, 0% (0/52) of those with other neurological diseases, and 0% (0/35) of healthy controls [6]. Among the OSMS patients, the antibody positivity rate was highest (55.6%) in OSMS patients with both LESCLs and MS-like brain lesions fulfilling the Barkhof criteria for MS [26], although NMO-IgG was originally described in patients with exclusively optic nerve and spinal cord lesions.

There are obvious discrepancies in the detection rates among the above-mentioned series in Japanese. The reasons for these may relate to differences in the subjects used: NMO versus OSMS patients with LESCLs; selected versus consecutive patients; northern versus southern Japanese patients that have been shown to have somewhat distinctive features in clinical phenotype by a recent nationwide survey [27,28]. They could also relate to the methods used: AQP4-transfected versus GFP–AQP4 fusion protein-transfected; fixed transfected cell specimens versus unfixed ones; 1:4 dilution versus 1:400 dilution. However, even in the studies done by the Mayo clinic, there are considerable differences in positivity rate, indicating that the difference is in part attributable to differences between subjects (Table 2) [3,5,6,19–23,29]. It remains to be elucidated whether the 30–70% of NMO patients who fulfilled the NMO diagnostic criteria and did not carry the antibodies are truly seronegative NMO patients or false negatives due to the low sensitivity of the assay.

5.2. Specificity

NMO-IgG has not been described in other inflammatory diseases in Westerners; however, 9% of MS cases in Lennon's

original series did have the antibodies [3]. To date, 5–15% of tested MS cases were found to be positive for NMO-IgG or anti-AQP4 antibody [3,5–7,20]. Even in the above-mentioned report by Pittock et al. [16], describing the occurrence of brain lesions in NMO patients, 10% of NMO-IgG-positive patients had brain lesions that were indistinguishable from MS lesions. This indicates the existence of considerable overlap between NMO and MS, which cannot be ignored.

6. Epitopes and titers of anti-AQP4 antibody

6.1. Epitopes

The finding that positive sera stained the cell surfaces of AQP4-transfected cells, but not the cytoplasm, suggests that patients' sera recognized the conformational epitopes of the molecule expressed on the cell surface [4,6]. Recently, by comparing the reactivity of NMO patients' sera against human, mouse and rat AQP4 proteins, which have several amino acid substitutions, the third extracellular loop of AQP4 was suggested to be the major epitope for AQP4 antibody in NMO patients [30]. AQP4 has two isoforms: the longer M1 isoform and the shorter M23 isoform lacking the N-terminal 22 amino acids. Only the presence of the M23 isoform induces formation of an orthogonal array of particles (OAPs) [31] and Nicchia et al. [32] reported that the NMO-IgG epitope is intrinsic in AQP4 assemblies into OAPs. All of the immunofluorescence studies mentioned above used the M1 isoform; however, in cells transfected with the M1 coding sequence, the M23 isoform was also detected because of leaky scanning for synthesis of the shorter M23 isoform from the second methionine [33].

6.2. Titers and seroconversion

Takahashi et al. [24] claimed that anti-AQP4 antibody titers showed a strong positive correlation with the spinal cord lesion length ($R = 0.9108$), while others have not found any correlation between the two parameters [6,7,34,35]. Although the NMO-IgG/anti-AQP4 antibody usually appears in the early course of the disease [36], seroconversion of NMO-IgG/anti-AQP4 antibody during the course of illness is observed in some patients [6,7]. This may indicate the possibility that the antibody is produced secondarily following tissue destruction in some patients, as seen in MS patients in whom various autoantibodies emerge during the clinical course; some of them target even neural antigens and are shown to be functional *in vivo* [37]. A recent report has indicated the emergence in animals with myelin-oligodendrocyte glycoprotein-induced EAE of anti-AQP4 antibody [38]. Thus, it will be crucial to examine whether antibodies recognizing conformational epitopes can be secondarily induced in myelin-sensitized EAE animals. If so, then it will be necessary to test whether such antibodies can modify the clinical course.

7. Clinical and neuroimaging characteristics of anti-AQP4 antibody-positive NMO patients and anti-AQP4 antibody-negative OSMS patients in Asians

Anti-AQP4 antibody-positive NMO patients demonstrate a higher age at onset, marked female preponderance (the male:female ratio is around 1:10), high frequency of relapses, severe visual disturbance due to severe optic nerve, high frequency of acute transverse myelitis (ATM) due to severe spinal cord damage, rare occurrence of secondary progression, LESCLs on spinal cord MRI, marked pleocytosis and neutrophilia in cerebrospinal fluid (CSF), and absence of oligoclonal bands (OB) in CSF, as compared with classical MS patients [5–7,17]. However, most conditions are also common to anti-AQP4 antibody-negative OSMS patients [6,7].

In Western MS series, spinal cord lesions usually span less than two vertebral segments and occupy less than one-half of a spinal cross-section, preferentially involving the peripheral white matter [39]. LESCLs extending over three vertebral segments are rarely seen in classical MS patients in Western populations; 3% according to Tartaglino [39]. However, in a recent study on Western populations, Bot et al. [40] reported a relatively high frequency (12.5%) of LESCLs in Western MS patients; 12.5% had long spinal cord lesions. On the other hand, in Asians, LESCLs are frequently observed in not only OSMS patients, but also CMS patients [41–44]. Indeed, LESCLs are seen in about half of OSMS cases and a quarter of CMS cases, reflecting the severe spinal cord damage seen in Asian MS patients. Detailed analyses of LESCLs on MRI disclosed that LESCLs in anti-AQP4 antibody-positive patients were located in the upper to middle thoracic cord, while those in anti-AQP4 antibody-negative OSMS patients were present throughout the cervical to thoracic cord [6]. In axial planes, the former most frequently involved the central gray matter while the latter showed a holocord involvement pattern [6]. By contrast, in anti-AQP4 antibody-negative CMS patients, both short and long spinal cord lesions preferentially involved the mid-cervical cord, presenting a peripheral white matter-predominant pattern [6].

Unexpectedly, anti-AQP4 antibody-positive NMO patients had a greater frequency of brain lesions than anti-AQP4 antibody-negative OSMS patients with LESCLs, further suggesting that the conditions are distinct [6,7]. Moreover, anti-AQP4 antibody-positive NMO patients showed less frequent responses to interferon beta (IFNβ)-1b than anti-AQP4 antibody-negative OSMS patients with LESCLs [6].

In our series, multiple logistic analyses disclosed that the emergence of the anti-AQP4 antibody was positively associated with only a higher relapse rate, but not LESCLs [6]. These observations collectively suggest that LESCLs are distinct according to anti-AQP4 antibody status and clinical phenotype, and that the mechanisms producing LESCLs are heterogeneous, even in cases with optic-spinal presentation,

namely AQP4 autoimmunity-related and -unrelated. In a randomized double-blind study of the efficacy of IFNβ-1b in Japanese patients with MS, the drug was found to be equally effective in CMS and OSMS patients [45]. Responsiveness to IFNβ-1b in OSMS patients is well explained by the presence of anti-AQP4 antibody-negative OSMS patients who can respond to the drug, suggesting the possibility that anti-AQP4 antibody-negative OSMS constitutes a spectrum of MS.

8. The nature of brain lesions in anti-AQP4 antibody-positive patients as determined by neuroimaging

Anti-AQP4 antibody-positive NMO patients occasionally develop huge brain lesions. Such extensive white matter lesions in anti-AQP4 antibody-positive NMO patients demonstrate high signal intensity on ADC maps and low or isointensity on diffusion-weighted MRI images (DWI), suggesting the nature of the lesions to be vasogenic edema [6,46]. On magnetic resonance spectroscopy (MRS), a high choline peak and a low n-acetyl aspartate (NAA) peak are observed, compatible with acute demyelination [6,46]. These findings strongly suggest that the nature of the lesions in anti-AQP4 antibody-positive MS patients is vasogenic edema. The frequent occurrence of spinal cord edema in the acute phase and its resolution in the convalescence phase following methylprednisolone pulse therapy in anti-AQP4 antibody-positive MS patients is also consistent with vasogenic edema. However, interestingly, even in such extensive brain lesions, gadolinium enhancement of the lesions is absent or scant [46,47], except for cases complicated with other systemic autoimmune diseases, suggesting preserved integrity of the blood–brain barrier (BBB) in this condition. Anti-AQP4 antibody may disturb AQP4 water channel function, thereby leading to inappropriate water transfer in the presence of intact BBB. Contrarily, Ito et al. [48] recently reported that multiple patchy enhancing lesions with blurred margins, described as "cloud-like enhancement", are found in 90% of NMO patients with contrast enhancement, suggesting breakdown of BBB in this condition. Therefore, the mechanism underlying relapse could be heterogeneous, even among individuals with anti-AQP4 antibody.

9. Background for NMO-IgG/anti-AQP4 antibody production

9.1. Autoimmune background

Relapsing NMO with anti-AQP4 antibody is frequently associated with other autoantibodies and autoimmune diseases, such as Sjögren syndrome, systemic lupus erythematosus, autoimmune thyroiditis, and myasthenia gravis, in Westerners [2,49]. Although in Asian OSMS patients such a high frequency of coexistent autoimmune disease has not been reported [1], other autoantibodies, such as SSA and SSB, as well as other autoimmune diseases, such as Sjögren syndrome, are frequently present even in Asian patients with anti-AQP4 antibodies [6,7,50–52]. Therefore, an autoimmune-prone background, especially heightened humoral autoimmunity, seems to be an important factor in the production of the anti-AQP4 antibody. We found that, among anti-AQP4 antibody-positive individuals, Th1 cell percentage showed a significant negative correlation with anti-AQP4 antibody titer, and that those with SSA/SSB antibody had significantly higher titers of anti-AQP4 antibody [7]. Therefore, high titer anti-AQP4 antibody seems to be produced in those with a heightened humoral autoimmune background, a Th2-prone condition. Considering that OSMS patients with low titer anti-AQP4 antibody showed similar clinical and immunological features to those of OSMS patients without the antibody, it may be possible that low titer anti-AQP4 antibody is secondary to severe tissue destruction [7].

9.2. HLA

In our series, the frequency of *HLA-DPB1*0501* was significantly increased in anti-AQP4 antibody-positive patients as compared with healthy controls, but not in anti-AQP4 antibody-negative OSMS patients with LESCLs [53]. In Caucasians, HLA-DRB3 was reported to be over-represented in NMO patients [54]. The HLA-DRB1*15 allele, which is the strongest disease susceptibility allele for MS in Westerners, is under-represented in NMO patients [23,54]. More recently, Isobe et al. [55] studied the epistatic interactions of HLA-DRB1 alleles in Japanese patients with MS and NMO; the frequency of HLA-DRB1*09 was decreased in both anti-AQP4 antibody-negative MS and anti-AQP4 antibody-positive patients with NMO spectrum disorders, while HLA-DRB1*12 increased the risk of anti-AQP4 antibody-positive NMO spectrum disorders. HLA-DRB1*09/15 decreased the risk of MS, whereas HLA-DRB1*12/15 increased the risk of NMO. These findings suggest the possibility that the anti-AQP4 antibody and NMO are induced with a certain genetic background; however, the genes associated with anti-AQP4 antibody production and NMO susceptibility could vary from race to race.

9.3. Paraneoplastic condition

NMO-IgG/anti-AQP4 antibody has been found in patients with malignancies, such as breast cancer, lung cancer, uterus cancer, thymoma, B cell lymphoma. Some showed NMO features while others had no NMO symptoms [56]. There are several reports describing NMO cases whose sera harbored anti-AQP4 antibody long before the onset of NMO [56,57]. AQP4 has now been added to the long list of onco-neural antigens. The occurrence of a healthy carrier state contradicts the primary proposed role of the anti-AQP4 antibody.

9.4. Infections

Wingerchuk et al. [2] reported that monophasic NMO is associated with preceding infection while relapsing NMO is associated with other autoimmunity. It was recently reported that 88% of parainfectious NMO is monophasic [58]. Hypercomplementemia and elevation of C-reactive protein are seen in anti-AQP4 antibody-positive patients with NMO spectrum disorders at relapse; however, such systemic inflammatory reaction is rare in classical MS [59]. Considering its relapsing nature, specific acute infection is less likely to play a role in causing relapsing NMO with anti-AQP4 antibody. However, we found that *Helicobacter pylori* infection is more frequent in anti-AQP4 antibody-positive individuals than anti-AQP4 antibody-negative CMS patients and healthy controls [60]. Chronic persistent infection may in part contribute to the development of NMO through molecular mimicry between bacterial AQP and human AQP4, and the effects of the infectious agents' products rendering the BBB leaky.

10. Immunohistopathological studies on NMO and Asian OSMS

In NMO, intense demyelination, a great loss of axons, perivascular lymphocytic infiltration, microglial proliferation and vascular proliferation are seen in optic nerve and spinal cord lesions; these can occasionally lead to cystic cavities in severely involved areas [9–11]. Astrocytosis is scarce in some necrotic lesions but considerable in others. On the other hand, the neuropathological features of MS in Asians are as follows: (1) preferential occurrence of lesions in the optic nerves and spinal cord; (2) necrotizing lesions with occasional cavity formation not only in the spinal cord and optic nerves but also in the cerebrum; (3) poor gliosis; and (4) poor perivascular cuffing in the necrotic form [12,61–63]. Perivascular cuffing and gliosis varied regionally. Spinal cord lesions were usually most severe in the lower cervical to the mid thoracic cord. Polymorphonuclear leukocyte infiltration was occasionally seen in severe lesions in Asian MS patients, but eosinophil infiltration, as described in Western NMO patients [64], was not reported in early [12,61–63] or more recent literature [65].

Ikuta et al. [63] compared MS pathology between 70 American and 75 Japanese autopsy cases and found that 47% of Japanese cases showed selective involvement of the optic nerves and spinal cord, while 13% of American cases also showed limited involvement of the optic nerves and spinal cord. Considering all of the reported evidence, it appears appropriate to assume that MS and NMO are not easily separable based on pathological findings alone.

More recently, Lucchinetti et al. [64] described perivascular immune complex deposition (IgM, IgG and C9neo) in a rim or rosette pattern. A similar finding has been reported by a Japanese group [66]. Misu et al. [66] reported extensive loss of AQP4 accompanied by decreased GFAP staining in active perivascular lesions where MBP staining was relatively preserved in postmortem Japanese NMO cases. Loss of AQP4 with MBP preservation was observed in 18 of 22 active inflammatory lesions, 11 of 25 active demyelinating lesions and 3 of 8 chronic active lesions, while it was not apparent in 12 chronic inactive lesions. Instead, losses of both AQP4 and MBP were found in 4 of 22 active inflammatory lesions, 13 of 25 active demyelinating lesions, 4 of 8 chronic active lesions and 7 of 12 chronic lesions. MBP loss with an AQP4 preservation pattern was seen in none of 22 active inflammatory lesions, 1 of 25 active demyelinating lesions, 1 of 8 chronic active lesions and 3 of 12 chronic inactive lesions. By contrast, in MS plaques, AQP4 was never lost but rather was upregulated, reflecting astrogliosis. Based on the presence of immunoglobulin and complement deposition in active perivascular lesions, Misu et al. [66] postulated that astrocytic impairment associated with the loss of AQP4 by humoral immunity is the primary event in NMO, suggesting a primary role for the anti-AQP4 antibody in NMO pathology. Roemer et al. [67] made similar observations regarding novel NMO lesions in the spinal cord and medullary tegmentum extending to the area postrema where the blood–brain barrier is absent.

However, even in Roemer's report [67], some MS plaques showed selective AQP4 loss. We [68] also found that, in some MS lesions, AQP4 was lost extensively far beyond the areas of myelin loss. Kobayashi et al. [69] reported an autopsied case of NMO showing preservation of AQP4 in the severe lesions in the spinal cord and medulla, and in the demyelinating lesions in the optic nerve. By pathological study of 11 autopsied NMO and NMO spectrum disorder cases, we also found that some demonstrated selective AQP4 loss while others showed preservation of AQP4, even in the acute lesions, and that even in identical individuals some lesions showed AQP4 loss while other lesions showed up-regulation of AQP4 [68]. Therefore, AQP4 down-modulation does not seem to be specific for NMO, and the mechanisms underlying AQP4 down-modulation could be heterogeneous.

11. Pathogenicity of NMO-IgG/anti-AQP4 antibody *in vitro* and *in vivo*

Sera and IgG from NMO patients with NMO-IgG/anti-AQP4 antibody induce astrocyte damage and death in primary culture only in the presence of complements [70–72], while in the absence of complement they do not affect AQP4 water channel function in astrocytes [32]. IgG containing anti-AQP4 antibody from NMO-IgG-seropositive NMO patients reproduces astrocyte loss *in vivo* only when myelin basic protein (MBP)-specific T cells are transferred to cause experimental autoimmune encephalomyelitis (EAE) [73–75]. However, when AQP4 antibody was injected into young rats with a leaky BBB, or after transfer of non-encephalitogenic T cells, it did not induce any disease or neuropathological alterations in the CNS [75].