resulting soluble N-terminal fragment is released into the culture medium (34).

The NgR coreceptor $p75^{NTR}$ transducing the signals from Nogo-66, MAG, and OMgp via NgR (16, 17) acts as a displacement factor that releases Rho from the Rho GDP dissociation inhibitor (Rho-GDI) (48). Neurons lacking $p75^{NTR}$ neither show RhoA activation nor exhibit neurite growth inhibition in the presence of myelin components (16). In general, $p75^{NTR}$ is expressed at high levels in neurons and glial cells during development, while its expression level declines to background levels in the adult CNS (49). However, $p75^{NTR}$ is reexpressed in the adult CNS following injury, ischemia, and inflammation (50). Previous studies by immunohistochemistry using a polyclonal anti-$p75^{NTR}$ antibody (G323A, Promega) showed that $p75^{NTR}$ is expressed on oligodendrocytes and microglia/macrophages in active MS lesions (51, 52). In contrast, the present study using the monoclonal antibody ME20.4 showed that $p75^{NTR}$ expression was limited in some regions such as substantia gelatinosa in the spinal cord, as described previously (53). A recent study showed that the antibody G323A exhibits a broad reactivity with numerous cross-reactive bands in PNS and CNS homogenates (54). Our observations support the view that NgR and $p75^{NTR}$ distribution does not always overlap in the CNS. NgR is identified in many cell types in the adult CNS that exhibit little or no $p75^{NTR}$ expression, whereas $p75^{NTR}$-expressing central cholinergic neurons in the medial septal nucleus do not express NgR (14). The depletion of $p75^{NTR}$ does not promote axonal regeneration after spinal cord injury (55), suggesting that an unidentified coreceptor for NgR might act as an alternative transducer of neurite growth-inhibitory signals (49). Another possibility exists that no alternative coreceptor is expressed in NgR-expressing cells where NgR acts as a nonsignaling receptor to take up an excessive amount of extracellularly released Nogo. Importantly, the NgR/$p75^{NTR}$ receptor complex is not required for mediating the neurite growth-inhibitory activity of the NAS domain of Nogo-A (56).

In conclusion, Nogo-A expression was upregulated in surviving oligodendrocytes, while NgR expression was enhanced in reactive astrocytes and microglia/macrophages in chronic active demyelinating lesions of MS, although the functional significance of these observations remains to be further investigated.

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Attack-related severity: a key factor in understanding the spectrum of idiopathic inflammatory demyelinating disorders

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Abstract

Understanding the spectrum of idiopathic inflammatory demyelinating disorders (IIDD) is a fundamental issue for the diagnosis and treatment of these disorders as well as for the approach to their pathogenesis. The spectrum of IIDD is usually classified according to clinical course and lesion distribution. We compared the demographic features, clinical characteristics, laboratory findings, and genetic backgrounds between 193 Japanese patients with and without *clinically* or *radiographically* fulminant attacks who all satisfied the diagnostic criteria for multiple sclerosis (MS). "Fulminant attacks" in the current study represent attack-related clinically or radiologically severe relapses but do not necessarily mean severe disability. Patients with fulminant attacks were clinically and immunogenetically distinct from those free of such attacks, and the previously described characteristics of the opticospinal form of MS (OSMS) or neuromyelitis optica (NMO) were mostly shared by patients with fulminant attacks. HLA profiles were similar among patients with fulminant attacks irrespective of the lesion distributions. The GG homozygous and G alleles of the CTLA4 gene A/G coding SNP at position 49 in exon 1 were significantly more common in patients with fulminant attacks than in those without. Attack-related severity may be an important factor if validated by prospective studies defining criteria and establishing relationships to disease course and treatment regimens.

Keywords: Multiple sclerosis; Immunogenetic background; Fulminant attack; Opticospinal MS; Neuromyelitis optica

1. Introduction

The spectrum of idiopathic inflammatory-demyelinating disorders (IIDD) of the central nervous systems (CNS) is usually classified based on two key factors, one is the clinical course, and the other is lesion distribution [1]. The spectrum includes monophasic, multiphasic, and progressive disorders ranging from highly localized forms of inflammatory demyelination to multifocal or diffuse variants; however, the nosology of the IIDD of the CNS remains confusing [2–5].

Multiple sclerosis (MS) is the most common IIDD and is well known to be a clinically, pathologically, and immunogenetically heterogeneous disorder [6–9]. Furthermore, the frequency of disorders within the spectrum varies with ethnic background [10,11]. The so-called opticospinal form of MS (OSMS) is one subtype of relapsing-remitting (RR) IIDD whose clinically determined lesions are restricted to the optic nerve and the spinal cord. The OSMS is relatively common in Japanese [10,12], and it appears to be identical to the relapsing neuromyelitis optica (NMO) reported in the west [13]. Apart from its unique pattern of lesion distribution, the OSMS or relapsing NMO has been emphasized to be clinically distinct among patients who satisfy the diagnostic criteria of MS. The condition is described as

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distinctively characterized by the following features: a relatively higher female/male ratio, attack-related severe myelitis and optic neuritis, central and extending cord lesions over three or more vertebral segments on magnetic resonance images (MRI), a lower frequency of OCB, and neuropathologically necrotic lesions [4,11,13–18]. Moreover, the OSMS was also reported to be immunogenetically distinct, and was described as an HLA-DPB1*0501-related autoimmune demyelinating disorder [18,19], while the OSMS was not associated with DRB1*1501, which is consistently associated with MS in Caucasians [20], and with the conventional form of MS (CMS) in Japanese [11,21].

Acute transverse myelitis (ATM) is a severe clinical syndrome among the IIDD spectrum, and can be associated with MS [10,12]. However, patients with ATM appear to constitute a distinct clinical subgroup among patients diagnosed with MS [22–24], and more than half of the patients were classified as OSMS [22]. Recently, we reported eight Japanese patients with a multiphasic inflammatory demyelinating disorder of the spinal cord, presenting as ATM, cerebrum, or brainstem involvement with fulminant clinical attacks [25]. All of the patients were women and lacked oligoclonal IgG bands (OCB) in the cerebrospinal fluid (CSF). The MRI findings of the cerebrum and brainstem were tumor-like or destructive. In that patient group, the frequencies of HLA-DPB1*0501 and DRB1*1501 were similar to those in the OSMS group. On the basis of shared clinical and radiological features, and immunogenetic backgrounds, we suggested that the patient group had a distinct IIDD linked to OSMS rather than to CMS [25]. On the other hand, it was recently suggested that the OSMS is not necessarily associated with DPB1*0501 and that mild OSMS may be associated with DRB1*1501, which is in turn associated with CMS [26]. These findings suggest that these HLA associations do not confer a unique pattern of lesion distribution, but rather are associated with the attack severity.

The aim of the present study was to verify the role of genetic background in determining the lesion distribution and attack-related severity of OSMS in Japanese patients, and to investigate whether attack-related severity should be considered as the third key factor in classifying the IIDD. For this purpose, we compared the demographic features, clinical characteristics, laboratory findings, and genetic backgrounds between patients with and without fulminant attacks who all satisfied the diagnostic criteria for MS. "Fulminant attacks" represent attack-related clinically or radiologically severe relapses which we consider to indicate the fulminancy of each attack or the expansion of each lesion, but do not necessarily mean severe disability. Based on our findings, we propose that the severity of each attack should be considered along with the lesion distribution and clinical course to fully understand the spectrum of IIDD or "MS" syndrome.

2. Patients and methods

2.1. Patients and controls

The study subjects were 193 unrelated Japanese patients, representing all patients diagnosed between 1986 and 2001 who had been clinically followed up for at least 1 year. All patients had exhibited two or more clinical attacks and objective clinical evidence of multiple lesions, and satisfied the diagnostic criteria for MS [27]. All patients showed a relapsing-remitting (RR) or secondary progressive (SP) course and were negative for anti-HTLV-1 antibody. Patients who were serologically positive for antinuclear antibody (ANA) on Hep-2 cells with any other evidence of collagen disease, thrombosis, or systemic vasculitis, were excluded from this study. Fifty-eight (30.1%) patients were classified as "fulminant MS (FIMS)" and fulfilled at least one of the following characteristics: (1) patients showed ATM according to the criteria described previously [22,28]. In brief, the criterion for the diagnosis of ATM was acutely developing paraparesis or tetraparesis affecting motor and sensory systems as well as sphincters with discrete sensory levels. (2) Visual acuity was irreversibly reduced to less than 20/200 after the initial attack of the optic nerve, (3) simultaneous bilateral visual loss, (4) attack-related respiratory failure or decreased consciousness, (5) generalized convulsive seizures, (6) spinal cord lesions were central and extending over three or more vertebral segments on MRI, or (7) there was a tumor-like or destructive appearance on MRI [25]. These criteria were selected to try to reflect the fulminancy of each attack and the expansion of each lesion in the current study. "Fulminant attacks" represent attack-related *clinically* or *radiographically* severe relapses but do not necessarily mean severe disability. Among the 58 FIMS patients, clinically determined lesions were confined to the optic nerve and spinal cord in 26 patients, and they were classified as OS-FIMS in the present study, while the remainder of the patients with FIMS was classified as nonOS-FIMS. Some of the aforementioned criteria such as multisegmental contiguous spinal cord involvement and severe optic neuropathy are consistent with OSMS as defined by previous reports [14,18,24]. However, nonOS-FIMS patients in the current study will never fall into the OSMS group because nonOS-FINS patients already had clinically apparent cerebellar, cerebral or brainstem lesions. The other 135 patients did not show any of the aforementioned seven features but exhibited chronic relapsing courses; the group was classified as non-FIMS. Among the 135 non-FIMS patients, clinically determined lesions were confined to the optic nerve and spinal cord in only four patients. These four non-FIMS patients were classified as nonFI-OSMS. A combined group of OS-FIMS and nonFI-OSMS was defined as OSMS in the present study.

The control group consisted of 154 unrelated healthy hospital staff members, all of whom had undergone annual medical examinations. All patients and control subjects were Japanese and were residents of Hokkaido, the northernmost island of Japan [12]. Written informed consent was obtained from all patients and control subjects before blood and CSF sampling.

2.2. Cerebrospinal fluid (CSF) analysis

Twenty of 26 OS-FIMS patients, 29 of 32 nonOS-FIMS patients, and 103 of 135 non-FIMS patients had at least one CSF examination during the acute phase. Since the various methods used to demonstrate oligoclonal IgG bands (OCB) can yield markedly different results, any technique that is used to detect OCB must be assessed by experienced observers who are blinded to the clinical data. Therefore, CSF and serum samples were tested at the Division of Clinical Chemistry of Vancouver Hospital and Health Sciences Center in Canada, using the methods described previously [6,9]. Eighteen OS-FIMS patients, 20 nonOS-FIMS patients, and 77 non-FIMS patients were tested for OCB by these procedures.

2.3. Studies for immunogenetic background

DNA typing analysis of HLA-DRB1 and-DPB1 alleles was performed with a nonisotopic oligotyping method using reverse dot blot hybridization (INNO-LiPA HLA kit; Innogenetics) [29]. When the discrimination was not clear by the reverse dot hybridization method, we reanalyzed by a standard polymerase chain-specific oligonucleotide probe (PCR-SSOP) method. In the present study, our interests were confined to DRB1*1501, DPB1*0501, DPB1*0301 and DRB1*0405 alleles because the first three alleles were reported to be associated with OSMS or CMS in Japanese [11,18,19,21,30–32], and the latter was reported to confer the OCB production in Japanese MS [6,9]. Other DRB1 and DPB1 polymorphisms were not used for the current analysis. Since T-cell apoptosis contributes to the resolution of CNS inflammation and clinical recovery from attacks of experimental autoimmune encephalomyelitis (EAE), an animal model of MS [33], the Apo-1/Fas and CTLA-4 molecules may be involved in the attack severity of MS. To verify the role of these genes in attack severity in MS, the Apo-1/Fas gene A/G SNP in the promoter at position-670, and the CTLA4 gene A/G coding SNP at position 49 in exon 1 were analyzed using the methods described previously [34,35].

2.4. Statistical analyses

2.4.1. Comparisons of demographic features, clinical characteristics and laboratory findings

Sex ratio, clinical course (RR or SP), MRI findings (presence or absence of periventricular ovoid lesions), and OCB positivity were compared using the chi-square test or Fisher's exact test. Age at onset, age at last clinical examination, disease duration, the expanded disability status scale of Kurtzke (EDSS), CSF leukocyte counts, and CSF protein levels were compared by analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD). These procedures were also used for comparison of the progression index (PI), which is a measure of accumulated disability over time (PI=EDSS/disease duration in years). To investigate whether these clinical subtypes confer OCB production, multiple logistic analysis was used adjusting for HLA-DRB1*1501 positivity, DRB1*0405 positivity and PVL on MRI, since these variables have been reported to contribute to OCB production in our population [9].

2.4.2. Comparisons of gene polymorphisms

Since the number of patients with nonFI-OSMS was small and the clinical features of nonFI-OSMS patients were not distinct among nonFIMS patients, the four patients with nonFI-OSMS were analyzed together as non-FIMS patients. The phenotype frequencies of DRB1*1501, DPB1*0501, and DPB1*0301 alleles were compared between OS-FIMS, nonOS-FIMS, non-FIMS, and controls, using the chi-square test or Fisher's exact test. To calculate the corrected P values (P_{corr}), the P values (uncorrected P, P_{uncorr}) were corrected for stratification according to the three MS subtypes in this study, and for the number of alleles compared, and P values were multiplied by a factor of 9. The Apo-1/Fas and CTLA-4 gene polymorphisms were compared using the chi-square test or Fisher's exact test. The relative risk (estimated as the odds ratios [OR]) and 95% confidence intervals (95%CI) were calculated.

Statistical analysis was performed using StatView version 5.0 (SAS institute). P values less than 0.05 were considered significant.

3. Results

3.1. Demographic and clinical features

Table 1 summarizes the clinical profiles of the three groups. The female/male ratios were similarly higher in OS-FIMS and nonOS-FIMS than in non-FIMS, although the differences did not reach significance. Age at onset was significantly higher in OS-FIMS than in nonOS-FIMS (p=0.0005) and non-FIMS (p<0.0001). Age at the last examination was also higher in OS-FIMS than in nonOS-FIMS (p<0.0001) and non-FIMS (p<0.0001). The number of OS-FIMS patients had decreased and those of nonOS-FIMS and non-FIMS patients had increased, and the distribution of the number of nonOS-FIMS patients by year of birth was strikingly similar to that of non-FIMS patients (Table 2). Progression indices were similar between OS-FIMS and nonOS-FIMS, while those of FIMS

Table 1
Clinical profiles of patients with OS-fulminant MS (OS-FIMS), nonOS-fulminant MS (nonOS-FIMS), and nonfulminant MS (non-FIMS)

	OS-FIMS (n=26)	nonOS-FIMS (n=32)	nonFIMS (n=135)
Men:women	1:5.5	1:4.2	1:2.2
Age at onset (years)*	40.7±14.0	30.4±13.1	27.2±9.7
Age at the last exam (years)**	54.0±14.9	38.7±13.7	38.0±11.1
Disease duration (years)	14.3±8.6	9.3±7.4	11.7±8.7
EDSS score	5.5±2.3	3.8±2.7	3.2±2.7
Progression index***	0.617±0.578	0.693±0.831	0.384±0.428
Secondary progressive (%)	0.0	9.4	28.1
Positive MRI findings (%)	7.7	46.9	94.8
	n=20	n=29	n=103
CSF WBC counts (mm^{-3})[a]	33.4±40.0	40.3±98.8	8.3±10.6
Pleocytosis>50/mm^3 (%)	25.0	24.1	0.0
CSF protein level (mg/dl)[b]	72.7±41.5	59.6±44.5	37.4±16.3
OCB positive rate	2/18 (11.1%)	3/20 (15.0%)	50/77 (64.9%)

Data are mean±S.D.
Opticospinal (OS, see text for the definition of the subtypes); expanded disability status scale of Kurtzke (EDSS), cerebrospinal fluid (CSF), white blood cell (WBC), oligoclonal IgG bands (OCB). Positive MRI findings indicate periventricular ovoid lesions on MRI (see text).
[a] p=0.0015.
[b] p< 0.0001 (ANOVA).
* p<0.0001.
** p<0.0001.
*** p=0.0049.

groups were significantly higher than that of non-FIMS (p=0.0437 and p=0.0038, respectively). Patients with FIMS showed mostly RR course, but 28.1% of 135 patients with non-FIMS had secondary progressive courses. Two of 26 (7.7%) OSMS patients, 15 of 32 (46.9%) nonOS-FIMS patients and 128 of 135 (94.8%) non-FIMS patients had periventricular ovoid lesions on MRI. The nonFI-OSMS group consisted of three females and one male, with age at onset being 16, 21, 32, and 34, respectively. The disease duration of the four nonFI-OSMS patients was 2, 4, 8, and 14 years, respectively, and all four patients had periventricular ovoid lesions on MRI.

Table 2
Number of patients according to the year of birth

Year of birth	OS-FIMS (n=26)	nonOS-FIMS (n=32)	nonFIMS (n=135)
1920–1929	6 (23.1%)	0 (0.0%)	0 (0.0%)
1930–1939	6 (23.1%)	2 (6.3%)	5 (3.7%)
1940–1949	5 (19.2%)	6 (18.8%)	18 (13.3%)
1950–1959	4 (15.4%)	4 (12.5%)	33 (24.4%)
1960–1969	2 (7.7%)	9 (28.1%)	34 (25.2%)
1970–	3 (11.5%)	11 (34.4%)	45 (33.3%)

3.2. CSF analyses

CSF profiles studied were similar between OS-FIMS and nonOS-FIMS, but they were significantly different between each FIMS group and non-FIMS (Table 1). White blood cell (WBC) counts in the CSF of OS-FIMS and nonOS-FIMS were significantly higher than that of non-FIMS (p=0.0271 and p=0.0012, respectively). Pleocytosis with >50 WBC/mm^3 was observed in 5 of 20 (25.0%) OS-FIMS patients, 7 of 29 (24.1%) nonOS-FIMS patients and none of 103 (0.0%) non-FIMS patients. The CSF protein levels were significantly higher in OS-FIMS and in nonOS-FIMS than in non-FIMS (p<0.0001 and p=0.0002, respectively). CSF OCBs were positive in 2 of 18 (11.1%) OS-FIMS patients, 3 of 20 (15.0%) nonOS-FIMS patients and 50 of 77 (64.8%) non-FIMS patients.

Univariate analyses showed that OCB positivity was associated with HLA-DRB1*1501 (p=0.0047; odds ratio=3.31; 95% CI=1.42–7.72) and with periventricular ovoid lesions (PVL) on MRI (p<0.0001; odds ratio=8.02; 95% CI=2.80–22.9). Conversely, the OCB positivity was negatively associated with HLA-DRB1*0405 (p=0.0044; odds ratio=0.297; 95% CI=0.126–0.699), with OSMS (p=0.0007; odds ratio=0.106; 95%CI=0.023–0.486) and with FIMS (p<0.0001; odds ratio=0.083; 95%CI=0.029–0.239). In a logistic regression model adjusted for these variables, however, OSMS and PVL were not associated with OCB poisitivity, while DRB1*1501, DRB1*0405 and FIMS were independent contributing factors to the production of OCB (Table 3). In the four nonFI-OS patients, pleocytosis or protein elevation was not found and OCB was positive in two (66.7%) out of three patients studied for OCB.

3.3. HLA phenotype frequencies of patients and controls

Table 4 shows the frequencies of DRB1*1501-, DPB1*0501- and DPB1*0301-positive patients. The DRB1*1501 and the DPB1*0301 alleles were more commonly found in non-FIMS alone. Although the corrected P value did not reach statistical significance, the frequency of the DPB1*0501 allele was higher in each patient group than in the control group. Differences in the frequencies of these three HLA alleles were not significantly

Table 3
Multiple logistic analysis for possible factors contributing to the production of oligoclonal IgG bands (n=114)

	P value	aOR	95% CI
DRB1*1501 (1 to –)	0.0357	2.97	1.08–8.18
DRB1*0405 (1 to –)	0.0046	0.236	0.087–0.641
PVL (1 to –)	0.5861	1.62	0.283–9.31
OSMS (1 to –)	0.4930	0.548	0.098–3.06
FIMS (1 to –)	0.0236	0.133	0.023–0.763

Adjusted odds ratio (aOR), periventricular lesions (PVL), a combined group of OS-FIMS and nonFI-OSMS (OSMS), opticospinal (OS), fulminant MS (FIMS, see text for the definition of the subtypes).

Table 4
Frequencies of DRB1*1501-, DRB1*0405-, DPB1*0501- and PB1*0301-positive patients and controls

	OS-FIMS (n=25)	nonOS-FIMS (n=31)	nonFIMS (n=130)	Controls (n=147)
Total patients and controls				
DRB1*1501	6 (24%)**	6 (19.4%)**	41 (31.5%)***	22 (15.0%)***
DPB1*0501	21 (84.0%)[a,b]	24 (77.4%)[b]	96 (78.8%)[c]	92 (62.6%)[b,c]
DPB1*0301	0 (0.0%)[d]	3 (9.7%)[d]	27 (20.8%)[e]	15 (10.2%)[e]
*Patients and controls lacking DPB1*0301 allele*				
	n=25	n=28	n=103	n=132
DPB1*0501	21 (84.0%)	24 (85.7%)	85 (82.5%)	86 (65.2%)

Opticospinal (OS), fulminant MS (FIMS, see text for the definition of subtypes).
[a] p_{uncorr}=0.0413, p_{corr}=0.3717 (odds ratio=3.14, 95% CI=1.02–9.62).
[b] p=0.5378.
[c] p_{uncorr}=0.0452, p_{corr}=0.4068 (odds ratio=1.69, 95% CI=1.01–1.82).
[d] p=0.2451.
[e] p_{uncorr}=0.0144, p_{corr}=0.1296 (odds ratio=2.31, 95% CI=1.67–4.56).
** p=0.6737.
*** p_{uncorr}=0.0010, p_{corr}=0.0090 (odds ratio=2.62, 95% CI=1.46–4.70).

different between OS-FIMS and nonOS-FIMS. Although the DPB1*0501-positive frequency appeared to be higher in OS-FIMS than in the other MS groups, the DPB1*0501-positive frequencies were almost similar in OS-FIMS (84.0%), nonOS-FIMS (85.7%) and non-FIMS (82.5%) in DPB1*0301-negative patients, which were higher than that of DPB1*0301-negative controls (65.2%).

3.4. Polymorphisms of Apo-1/Fas and CTLA-4 genes

The profile of the Apo-1/Fas gene polymorphism was not different between OS-FIMS, nonOS-FIMS, non-FIMS, and controls (data not shown). The CTLA-4 G allele was positive in 24 of 25 (96.0%) OS-FIMS patients, 27 of 29 (93.1%) nonOS-FIMS patients, 99 of 119 (83.2%) non-FIMS patients, and 128 of 154 (83.1%) controls. The CTLA-4 A allele was positive in 14 of 25 (56.0%) OS-FIMS patients, 11 of 29 (37.9%) nonOS-FIMS patients, 81 of 119 (68.1%) non-FIMS patients and 91 of 154 (59.1%) controls. The genotype frequencies and allele frequencies were similar between OS-FIMS and nonOS-FIMS (data not shown), whereas the GG genotype and G allele were significantly more common in FIMS than in non-FIMS (Table 5).

Table 5
CTLA-4 gene polymorphism in patients and controls

	FIMS (n=54)	nonFIMS (n=119)	Controls (n=154)
Genotype frequency			
G/G	29 (53.7%)	38 (31.9%)	63 (40.9%)
G/A	22 (40.7%)	61 (57.3%)	65 (42.2%)
A/A	3 (5.6%)	20 (16.8%)	26 (16.9%)
Allele frequency			
G	80 (74.1%)	137 (57.6%)	191 (62.0%)
A	28 (25.9%)	101 (42.4%)	117 (38.0%)

Fulminant MS (FIMS), CTLA-4 gene polymorphism; exon 1 A/G cSNP.
FIMS vs. nonFIMS; p=0.0115 (genotype frequency), p=0.0033 (allele frequency).
FIMS vs. controls; p=0.0750 (genotype frequency), p=0.0236 (allele frequency).

4. Discussion

In the present study of Japanese patients who satisfied the diagnostic criteria of MS, we found the following: (1) apart from the unique pattern of lesion distribution, CSF characteristics and progression index of OSMS patients with fulminant attacks (OS-FIMS) were very similar to those of non-OSMS patients with fulminant attacks (nonOS-FIMS), but distinct from patients without fulminant attacks (non-FIMS). (2) The pattern of lesion distribution restricted to the optic nerve and the spinal cord was not independently associated with OCB positivity, but FLMS was less frequently associated with the presence of oligoclonal bands. (3) The frequencies of DRB1*1501, DPB1*0501 and DPB1*0301 alleles were not significantly different between OS-FIMS and nonOS-FIMS, and the DRB1*1501 and the DPB1*0301 alleles were more commonly found in non-FIMS alone. (4) The GG homozygous and G allele of CTLA-4 gene exon 1 were significantly more common in FIMS than in non-FIMS. (5) The number of OS-FIMS patients had decreased and those of nonOS-FIMS and non-FIMS patients had increased, and the distribution of the number of nonOS-FIMS patients by year of birth was strikingly similar to that of non-FIMS patients. (6) OSMS patients without fulminant attacks (nonFI-OSMS) were rare (2.1% of patients studied) and their clinical features appeared similar to those of non-FIMS patients. These findings indicate that nonOS-FIMS is clinically and immunogenetically linked to the OS-FIMS, but is very distinct from non-FIMS, which is irrelevant to the differences in lesion distribution. We propose that there is a need for redefining the important and unique characteristics of OSMS or relapsing NMO, and the spectrum of IIDD or "MS" syndrome.

Despite numerous reports on OSMS or relapsing NMO, the definitions of these conditions have not been uniform among investigators. The optic nerve and spinal cord are frequently involved in MS in general, and it is easier to detect small lesions in the optic nerve or spinal cord than in the cerebrum or cerebellum in the clinical setting. Furthermore, patients with clinically determined OSMS may have asymptomatic lesions of the cerebrum, cerebellum or brainstem, even at onset or at a later clinical phase. We have been further confused because it was reported that the OSMS had decreased in frequency and become milder in its presentation [36,37]. Should previous reports have emphasized that an attack-related severe involvement is one of the key characteristics of the OSMS as well as NMO? On the other hand, patients with cerebral, cerebellar, or brainstem lesions may also have characteristic features considered to be unique to the OSMS or NMO, and we recently reported eight such representative patients [25]. The patient group was clinically and immunogenetically similar by the OSMS, and, in the current study, we obtained almost the same results from an expanded data set with respect to attack severity. Clinical features, CSF findings, MRI findings of the spinal cord, and clinical courses were very similar between OS-FIMS and nonOS-FIMS, but were significantly different between FIMS and non-FIMS, irrespective of the lesion distribution. Furthermore, a lower rate of OCB positivity was not an independent unique characteristic of the OSMS but seemed to be of FIMS.

With regard to HLA profiles, recent reports have indicated the important role of the DRB1*1501, DPB1*0301, and DPB1*0501 alleles in developing MS in Japanese [11,18,19,21,30–32]. The present study revealed that the frequencies of DPB1*0501 were similarly higher in OS-FIMS, nonOS-FIMS, and non-FIMS than in controls among DPB1*0301-negative subjects, that the DRB1*1501 and the DPB1*0301 alleles were more commonly found in non-FIMS alone, and that no HLA allele studied was uniquely associated with OS-FIMS. Furthermore, the results of recent analysis of patients with "pure OSMS" who had consistently normal brain MRI scans, suggested that OSMS is not necessarily associated with DPB1*0501 and that mild OSMS may be associated with DRB1*1501, which is associated with CMS [26]. Based on clinical features, radiological findings, CSF findings, and immunogenetic backgrounds, patients with fulminant attacks should be delineated from those without fulminant attacks. Non-FIMS may be reclassified into FIMS after longer observation, but FIMS can never change to non-FIMS by definition, therefore, longer observation may more precisely define the difference between patients with and without fulminant attacks.

Several possible mechanisms for the difference in attack-related severity should be considered. We investigated CTLA-4 gene exon 1 A/G SNP since the CTLA-4 gene encodes a T-cell surface molecule that binds to the B7 molecule on antigen presenting cells to deliver a negative signal to T-cells, and thereby mediates apoptosis. CTLA-4 expression on T-cell thus might influence the course of an ongoing immune process [38]. We found that the GG homozygous and G alleles were more common in FIMS than in non-FIMS. These results are compatible with previous findings that homozygosity for AA was associated with significantly increased expression of both cell-surface CTLA-4 after cellular stimulation and of CTLA-4 mRNA in nonstimulated cells [39]. Our results suggest that dysregulation of the CTLA-4-driven downregulation of T-cell activation could influence the attack severity in a non-antigen specific manner. Interestingly, the homozygous genotypes of the GG and G allele of the CTLA-4 exon 1 are more common in Asian populations than in Western populations (Table 6) [34,40–43]. The higher prevalence of the OSMS with fulminant attacks in Asian populations may be in part accounted for by the high frequency of the CTLA-4 G allele. Our findings may indicate that the attack-related severity is, at least in part, controlled by host genetic backgrounds. Another possibility for the difference in attack severity is antigen-specific attack against both myelin and axons, although no common antigen targeted in MS was identified. Other regulatory cells or cytokines may also contribute to the occurrence of fulminant attacks. Basic research is required into attack severity to understand the pathomechanism of MS and IIDD.

Our data on distribution of the categorized subjects by year of birth appears to support previous reports of a declining incidence of OSMS [36,37]. A previous study on changes in the clinical phenotypes of MS during the past 50 years in Japan suggested that some environmental factors have contributed the declining the incidence of OSMS in Japanese. Conversely, the current study revealed that year of birth was different between OS-FIMS patients and nonOS-FIMS patients, whereas it was strikingly similar between nonOS-FIMS and non-FIMS patients irrespective of attack-related severity. The incidences of nonOS-FIMS and non-FIMS patients have increased. These findings may indicate that the distribution of lesions restricted to the optic nerve and spinal cord in the OSMS is influenced by environmental factors while genetic factors are relevant to the attack fulminancy irrespective of lesion distributions.

There has been tremendous progress in the immunomodulatory treatment of MS and other IIDD during recent

Table 6
Genotype frequencies of CTLA-4 in controls in different ethnic groups (%)

Genotype	Japanese[a] (n 200)	Chinese[b] (n=86)	Swedish[c] (n=237)	Dane[b] (n=125)	Norwegian[d] (n=271)
G/G	39	52	13	18	21
G/A	44	40	52	54	44
A/A	17	8	35	28	35

[a] Yanagawa et al. [42].
[b] Rasmussen et al. [43].
[c] Ligers et al. [41].
[d] Harbo et al. [40].

years, but not all patients with the disorders respond well to the available treatment, probably due, in part, to the disease heterogeneity [44]. For example, azathioprine and prednisone were suggested to reduce relapse frequency in relapsing NMO and protect optic nerve and spinal cord function more effectively than interferons [45]. If the nonOS-FLMS links to OSMS, which appears to be identical to the relapsing NMO [13], as suggested in the present study, these strategies may be appropriate for the treatment of MS with "fulminant attacks". Understanding the spectrum of IIDD is a fundamental issue for the diagnosis and therapy of these disorders as well as for the approach to their pathogenesis. Although there may be criticisms against the term of "fulminant" MS and "attack-related severity" and there may be more appropriate selection criteria than those we used in the current study, the fulminancy of each attack and the expansion of each lesion, which, here we call "attack-related severity" may be a third important factor, after lesion distribution and clinical course, to consider when addressing the spectrum of IIDD or "MS" syndrome, and to advance treatment strategies for these disorders. Prospective studies are warranted to validate the criteria for determining a fulminant attack as well as possible alternative strategies for MS with "fulminant attacks".

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CTLA-4 gene polymorphism is not associated with conventional multiple sclerosis in Japanese

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Abstract

We investigated the polymorphisms of exon 1 (149A/G) and promoter (−318C/T and −651C/T) regions of the CTLA-4 gene in 133 Japanese patients with conventional/classical multiple sclerosis (MS) and 156 healthy controls. Patients with optico-spinal MS (OSMS) or atypical clinical attacks were excluded from the study. There was no significant difference in the distribution of polymorphisms between patients and controls. Furthermore, there were no associations between polymorphisms and clinical characteristics, such as age at onset, disease prognosis, and HLA profiles. Our results suggest that CTLA-4 gene polymorphisms are neither conclusively related to susceptibility nor to the clinical characteristics of MS, especially in Japanese patients with conventional/classical form and clinical features identical to those of their counterparts in Western countries.

Keywords: CTLA-4 gene polymorphism; HLA-DRB1*1501; Multiple sclerosis; Disease susceptibility; Disease prognosis; Japanese

1. Introduction

Multiple sclerosis (MS) is considered as an autoimmune disease, and susceptibility to this condition is controlled by multiple genes and environmental factors (Vyse and Todd, 1996). Despite evidence for a strong genetic influence, a weak major histocompatibility complex (MHC) association is the only consistently observed genetic feature in MS (Hillert, 1994; Compston et al., 1995), and recent genome wide linkage studies demonstrated that MS follows a polygenetic trait with multiple loci (Ebers et al., 1996). The genes involved in polygenic diseases like MS are not easily identified because clinical manifestation requires several disease-associated alleles of several genes rather than one specific mutation. The analysis of multifactorial diseases like MS is further complicated by the fact that functional differences of known polymorphisms have not yet been identified.

CTLA-4 gene is a strong candidate gene for involvement in autoimmune diseases because it plays an important role in the termination of T cell activation (Waterhouse et al., 1995; Ueda et al., 2003). The CTLA-4 gene is located on chromosome 2q33 region, a region recognized as a candidate locus by linkage genome scan (Ebers et al., 1996). Several polymorphisms in the CTLA-4 locus have been reported, and several studies have addressed the potential role of single nucleotide polymorphism (SNP) in exon 1 (+49A/G), a microsatellite (AT)n marker at position 642 of exon 4, and SNPs in the promoter regions (−318C/T and −651C/T) of the CTLA-4 gene in susceptibility to MS with different results in different ethnic groups (Harbo et al., 1999; Ligers et al., 1999; Andreevskii et al., 2002; Rasmussen et al., 2001; Dyment et al., 2002; Maurer et al., 2002; van Veen Tineke et al., 2003; Kantarci et al.,

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2003). Interactions between CTLA-4 gene and HLA DR2 in the development of MS were also reported (Rasmussen et al., 2001; Alizadeh et al., 2003). In contrast, we previously found no association between exon 1 (+49A/G) SNP and MS in 74 Japanese patients and 93 controls, although the polymorphism was suggested to modulate the disease (Fukazawa et al., 1999). In our previous study, however, only exon 1 (+49A/G) SNP was investigated, and the subjects of the study included patients with clinically or paraclinically atypical attacks, as described previously (Fukazawa et al., 1999; 2004).

The aims of the present study were to analyze the relationships of three CTLA-4 gene polymorphisms [exon 1 (+49A/G) and promoter regions (−318C/T and −651C/T)] with disease onset and disease prognosis in an expanded data set of 133 Japanese patients with MS. We also investigated whether the CTLA-4 gene polymorphism interacts with HLA-DRB1*1501 in the development of MS. Patients with optico-spinal MS (OSMS) were excluded from the present study. Patients with atypical clinical or paraclinical findings (Fukazawa et al., 2004) were also excluded, and thus, the clinical features of the selected patients were identical to those in Western countries. All subjects studied were residents of Hokkaido, the northernmost island of Japan.

2. Patients and methods

2.1. Subjects

The study subjects were 133 unrelated Japanese patients with conventional/classical MS (CMS) who met the inclusion and exclusion criteria described below. All patients exhibited two or more clinical attacks and had objective clinical evidence of multiple lesions without any evidence of other disorders. They also fulfilled the diagnostic criteria for MS (Poser et al., 1983; McDonald et al., 2001). All patients showed a relapsing remitting or secondary progressive course. Patients with neuromyelitis optica (NMO) or optico-spinal MS (OSMS) were excluded. Patients with clinically or paraclinically atypical attacks were also excluded because they have been reported to be a clinically and immunogenetically distinct subtype among patients with diagnosis of MS (Fukazawa et al., 2003; 2004). The definitions of OSMS and atypical attacks were described previously (Yamasaki et al., 1999; Fukazawa et al., 2000; 2003; 2004). Therefore, in the current study, all patients studied were classified as having "conventional/classical MS (CMS)" with involvement of multiple sites in the CNS, including the cerebrum, cerebellum, or brainstem, with clinical features similar to those observed in Western countries (Fukazawa et al., 2000; 2004; Weinshenker, 2003). Among 133 patients studied, 61 patients had participated in our previous study and were analyzed for exon 1 A/G polymorphisms (+49; Fukazawa et al., 1999). The control group comprised 156 healthy Japanese volunteers. All study participants were Japanese and were resident of Hokkaido, the northernmost island of Japan. Their ancestors were from various parts of Japan, since Hokkaido was first reclaimed around 1870. The native inhabitants of Hokkaido are said to be the Ainu tribe, but this remains a controversial issue partly due to lack of information on the origin of this tribe. Informed consent was obtained from each individual in writing at the time of blood sampling.

2.2. Analysis of CTLA-4 polymorphism and HLA-typing

A blood sample was obtained, and high molecular weight DNA was extracted from peripheral blood cells. Exon 1 A/G polymorphisms (+49) were determined using the method described previously (Fukazawa et al., 1999). Genotypes at polymorphic sites −318 and −651 in the promoter region of the CTLA-4 gene were determined by polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP). The genotype at position −318 in the promoter region was identified as described previously (Rasmussen et al., 2001). The −651 SNP was detected using mismatch primers with sequences 5′-ttttatggacggctctaatctc-3′ and 5′-agaaaaaaatcacaagaaataaactgaaaatagc-3′. The amplified products were digested with *Msp*I (Boehringer Mannheim, Mannheim, Germany) and analyzed on 3% agarose gel. The C allele corresponds to the presence of two 42- and 144-bp fragments generated by *Msp*I digestion, and the T allele corresponds to the 186-bp uncleaved fragment with no *Msp*I site. Exon 1 (+49) and the promoter (318) dimorphisms were determined by using the protocol described previously (Harbo et al., 1999). We used primers with sequences 5′-TCTTTTCCGCCTATTTTCAGTT-3′ and 5′-CCCTGGAATACAGAGCCAGC-3′, and the amplified products were treated with the restriction enzymes *Tse*I and *Mse*I. On agarose gel electrophoresis, the haplotype combination of the polymorphic positions at +49 and 318 was identified by the 626-bp fragment (corresponding to the 318C, +49 A haplotype), 530-bp fragment (corresponding to the 318 T, +49 A haplotype), 460-bp fragment (corresponding to the 318 C, +49 G haplotype), and 365-bp fragment (corresponding to the 318 T, +49 G haplotype).

DNA typing of DRB1 alleles was analyzed by the nonisotopic oligotyping method using reverse dot blot hybridization. When the discrimination was not clear by the reverse dot blot hybridization method, we used the standard PCR-specific oligonucleotide probe (PCR-SSOP) method.

2.3. Disease prognosis

MS severity was defined according to the expanded disability status scale of Kurtzke (EDSS), progression index (PI), and ranked severity score (RSS). PI was calculated as a

measure of accumulated disability over time (PI=EDSS/disease duration in years), and then we determined RSS for patients with disease duration longer than 5 years (Rodriguez et al., 1994; Weinshenker et al., 1997). The RSS describes disability, and the score is inversely related to the EDSS. Since disease progression in the early stages of MS may be variable and not reflect the potential for prognosis in the longer term, we analyzed CTLA-4 gene-polymorphism associations with severity in patients with disease duration longer than 5 years.

2.4. Statistical analysis

Allele frequencies and genotype frequencies of the CTLA-4 gene were compared between MS patients and controls, using the chi-square test or Fisher's Exact Test. Phenotype frequencies of HLA-DRB1*1501 were also examined for association with conventional MS. To investigate the potential interactions between the DRB1*1501 allele and the CTLA-4 alleles, allele frequencies and genotype frequencies of the CTLA-4 gene were compared separately among DRB1*1501-positive and -negative subjects. The p value was multiplied by 2 to correct for stratification according to DRB1*1501 status. We analyzed CTLA-4 gene-polymorphism associations with PI and RSS using Kruskal–Wallis test. Statistical analysis was performed with StatView version 5.0 (Abacus Concept, Berkeley, CA).

3. Results

3.1. Clinical profile of patients studied

Patients consisted of 43 men and 90 women (male/female ratio=0.48). The mean age at blood sampling was 35.0 years (S.D.=10.7; range: 14–67). The mean age at onset was 27.1 years (S.D.=9.6; range: 4–57). The mean duration of disease was 11.5 years (S.D.=8.7; range: 1–37). The EDSS ranged from 0.0 to 9.5 (mean=3.0; S.D.=2.6). Clinical features of these cases with conventional MS were quite similar to those of MS in Western populations (Weinshenker, 2003; Fukazawa et al., 2004). The control subjects were Japanese of ethnic background similar to that of the study group and consisted of 52 men and 104 women (male/female ratio=0.50), and their mean age at blood sampling was 33.5 years (S.D.=9.2; range: 20–58). The sex ratio and the mean age of patients were not significantly different from those of the control.

3.2. CTLA-4 gene polymorphisms

The genotype and allele frequencies of CTLA-4 gene exon 1 +49 SNP, promoter −318 SNP, and the haplotypes of the 2 alleles are shown in Tables 1 and 2. In control subjects, the genotype frequencies conformed to Hardy–Weinberg expectations. The distributions of CTLA-4 exon 1 +49 and promoter −318 genotypes and allele frequencies were similar in CMS patients and controls. The frequencies of the CTLA-4 haplotypes studied were also similar in patients and controls (Table 1). Only the T allele was found at promoter −651 position in our Japanese population without polymorphism.

3.3. HLA-DRB1*1501 positivity and its interaction with CTLA-4 gene

A significantly higher frequency of DRB1*1501 was found in MS patients (p=0.0033; odds ratio=2.29; 95%CI=1.32–3.98) than in controls. Among HLA-DRB1*1501-positive subjects, the CTLA4 exon 1 +49 genotypes and haplotypes were almost equally distributed between patients and controls (Table 1). The frequency of C/T genotype at promoter −318 was higher in DRB1*1501-positve patients (16/43; 37.2%) than in DRB1*1501-positive controls (4/27; 14.8%), but the difference was not significant (corrected p=0.139; Table 1). Allele frequencies of CTLA-4 exon 1 +49 and promoter −318 SNP were also equally distributed between patients and control subjects among HLA-DRB1*1501-positive subjects (Table 2).

3.4. Clinical characteristics and CTLA-4 polymorphism

Among the 133 conventional MS patients, there were no associations between exon1 +49, promoter −318 polymorphisms, and haplotypes and clinical characteristics. The clinical characteristics were age at disease onset and clinical course (relapsing and remitting or secondary progressive; data not shown). Disease prognosis was analyzed for 97

Table 1
Distribution of CTLA-4 exon 1 +49 and promoter −318 genotypes and haplotypes in conventional MS patients and controls

	Patients		Control	
	Total	DRB1*1501 +	Total	DRB1*1501 +
	n=133	n=43	n=156	n=27
Exon 1 +49 genotypes				
AA	23 (17.3)	8 (18.6)	29 (18.6)	5 (18.5)
AG	69 (51.9)	23 (53.5)	66 (42.3)	14 (51.9)
GG	41 (30.8)	12 (27.9)	61 (39.1)	8 (29.6)
Promoter −318 genotypes				
CC	100 (75.2)	27 (62.8)	124 (79.5)	22 (81.5)
CT	32 (24.1)	16 (37.2)	28 (17.9)	4 (14.8)
TT	1 (0.8)	0 (0.0)	4 (2.6)	1 (3.7)
Haplotypes				
CA	81 (30.5)	23 (26.7)	88 (28.2)	18 (33.3)
CG	151 (56.8)	47 (54.7)	188 (60.3)	30 (55.6)
TA	34 (12.8)	16 (18.6)	36 (11.5)	6 (11.1)
TG	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

Numbers in parentheses represent percentage of subjects.
No significant differences were detected between patients and control.

Table 2
Allele frequencies of CTLA-4 exon 1 +49 and promoter −318 SNP in patients with conventional MS and control subjects by DRB1*1501 status

	Patients		Control	
	Total	DRB1*1501+	Total	DRB1*1501+
	n=266	n=86	n=312	n=54
Exon 1 +49 genotypes				
A allele	115 (43.2%)	39 (45.3%)	124 (39.7%)	24 (44.4%)
G allele	151 (56.8%)	47 (54.7%)	188 (60.3%)	30 (55.6%)
Promoter −318 genotypes				
C allele	232 (87.2%)	70 (81.4%)	276 (88.5%)	48 (88.9%)
T allele	34 (12.8%)	16 (18.6%)	36 (11.5%)	6 (11.1%)

SNP—single nucleotide polymorphism.
No significant differences were detected in allele frequencies between the two groups.

patients, and CTLA-4 polymorphisms were not associated with prognosis indicated by PI or RSS (data not shown).

4. Discussion

We found no differences in CTLA-4 polymorphisms between patients and controls irrespective of DRB1*1501 status. Furthermore, our present results indicated that CTLA-4 polymorphisms are not associated with the disease prognosis.

Several studies have examined the association between CTLA-4 gene polymorphisms and MS. There was no evidence for CTLA-4 as a susceptibility gene in Caucasians from Denmark (Rasmussen et al., 2001), Canada (Dyment et al., 2002), Germany (Maurer et al., 2002), Netherlands (van Veen Tineke et al., 2003), and Russia (Andreevskii et al., 2002). On the other hand, exon 1 +49A/G genotype was associated with MS in Norwegian (Harbo et al., 1999), and GG genotype in Swedish populations (Ligers et al., 1999). Furthermore, an American study showed the association of MS with the homozygous common haplotype A (+49)/C (−318)/AT_8 (−642) (Kantarci et al., 2003). Furthermore, the interactions of HLA-DR2 with A (+49)/C (−318) haplotype and C/T SNP in the promoter region (−651) of the CTLA-4 gene in the development of MS were reported in Chinese (Rasmussen et al., 2001) and European Caucasians (Alizadeh et al., 2003), respectively. In addition, polymorphisms have been suggested to influence the clinical course or disability (Harbo et al., 1999; Maurer et al., 2002). Therefore, the role of CTLA-4 gene polymorphisms seems to differ among various ethnic groups. On the other hand, our previous study on Japanese population showed no association between MS and CTLA-4 gene exon 1 A/G +49 polymorphism but suggested that the polymorphism may modulate the disease (Fukazawa et al., 1999). The present study again failed to detect associations between CMS and CTLA-4 polymorphisms irrespective of HLA-DRB1*1501 status. Accordingly, we conclude that CTLA-4 gene does not correlate with risk of CMS in Japanese. The present study could not confirm that CTLA-4 gene polymorphisms modulate the disease process. In our previous study, the number of subjects studied was too small to investigate patient prognosis. Furthermore, our previous study included patients with clinically or radiologically atypical attacks, and those atypical patients were recently suggested to constitute a distinct subgroup among Japanese patients with a diagnosis of MS (Fukazawa et al., 2003; 2004). Therefore, CTLA-4 gene is less likely to influence the prognosis of patients, especially those with conventional/classical form of MS, whose clinical features are identical to those of their Western counterparts. In contrast, previous studies implicated CTLA-4 gene in susceptibility to diabetes mellitus (Marron et al., 1997; Van der Auwera et al., 1997), and one of these studies indicated that the transmission of SNPs in regulatory regions was crucial in this process (Marron et al., 1997). Accordingly, exclusion of the role of CTLA-4 may require a dense SNP association study. Furthermore, since MS is clinically, pathologically, and immunogenetically heterogeneous, the possible relationships between clinical subtypes and the role of CTLA-4 polymorphisms should be further investigated in larger populations.

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PAPER

Chronic inflammatory demyelinating polyneuropathy: decreased claudin-5 and relocated ZO-1

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Objectives: To clarify the dynamics of molecules composing the blood–nerve barrier (BNB) in inflammatory neuropathies.
Methods: The expression of four tight junction (TJ) proteins—claudin-1, claudin-5, occludin, and ZO-1—was analysed immunohistochemically in sural nerve biopsy specimens obtained from patients with chronic inflammatory demyelinating polyradiculoneuropathy (CIDP).
Results: Claudin-1 was detected only in perineurial cells, whereas claudin-5 was present in endothelial cells, irrespective of vessel location or size. Occludin and ZO-1 were found in perineurial cells, in addition to some epineurial and endoneurial endothelial cells. In CIDP, percentages of endoneurial small vessels immunoreactive for claudin-5 were significantly decreased, as were ZO-1 immunoreactive endoneurial small vessels, with staining localised to interfaces between cells. Claudin-1 and occludin immunoreactivity did not differ appreciably between the neuropathies examined.
Conclusions: The downregulation of claudin-5 and altered localisation of ZO-1 seen in CIDP specimens may indicate that BNB derangement occurs in inflammatory neuropathies. Further investigation of TJ molecules may suggest new treatments based on properties of the BNB.

Endothelial cells in the adult central nervous system (CNS) and also in the peripheral nervous system (PNS) are coupled by tight junctions (TJ) that resemble those of epithelial barriers[1 2] and show extremely low permeability. Breakdown of these TJs in the blood–nerve barrier (BNB) may allow antimyelin antibodies and various inflammatory cytokines to enter peripheral nerve tissues, exacerbating peripheral nerve injury in inflammatory neuropathies, including Guillain-Barré syndrome[3] and chronic inflammatory demyelinating polyneuropathy (CIDP). The development of new therapeutic strategies to restore BNB function based on the properties of TJs has long has been awaited. Understanding of the molecular basis of TJs has been increasing since 1993, when occludin, a protein with four transmembrane domains, was discovered.[4] Occludin has also been detected in endothelial cells forming the blood–brain barrier (BBB).[5] Occludin deficient cells have been found to form morphologically normal TJs.[6] However, a new family of TJ proteins, claudins, has received attention more recently. Claudins also have four transmembrane domains, but do not show homology to occludin.[7 8] Among claudin family members, claudin-1, claudin-5, and claudin-11 have been demonstrated in the brain.[7 8] Because claudin-11 is expressed only in oligodendrocytes,[9] and presumably is not related to endothelial barriers, we focused on claudin-1 and claudin-5 as molecules of interest concerning the barrier system in both the CNS and PNS. In our present study, using immunohistochemistry, we analysed the expression of four representative TJ proteins—claudin-1, claudin-5, occludin, and ZO-1—in the human PNS using sural nerve biopsy specimens. We examined the expression of these molecules in PNS disorders in which BNB breakdown is believed to have an important pathogenetic role.

MATERIALS AND METHODS

Patients

Sural nerve biopsy specimens obtained from 25 patients including 10 from patients with CIDP (eight men and two women; age range, 13–64 years; mean, 44.3) were studied. Informed consent was obtained from each patient. The study complied with the ethical guidelines of Tokyo Medical and Dental University. Diagnoses were based on detailed clinical and electrophysiological investigations of the patients, in addition to the pathological examination of sural nerve specimens, including toluidine blue stained semithin sections and teased fibre preparations. CIDP was diagnosed according to standard criteria,[10] and all 10 patients were categorised as "definite" CIDP. Table 1 lists the clinical features and immunostaining results in the patients with CIDP. Other nerve biopsy specimens studied as disease controls included those obtained from six patients with vasculitic neuropathy associated with the Churg-Strauss syndrome (three men and three women; age range, 44–67 years; mean, 57.2); six with hereditary neuropathy (three men and three women; age range, 17–61 years; mean, 45.5; five with type I Charcot-Marie tooth disease and one with hereditary sensory and autonomic neuropathy type II); and four with nutritional neuropathy resulting from vitamin B_1 deficiency (three men and one woman; age range, 23–62 years; mean, 45.8). Specimens were snap frozen and stored at −80°C until use.

Immunohistochemical techniques

Rabbit polyclonal antibodies against human claudin-1, claudin-5, occludin, and ZO-1 were purchased from Zymed (South San Francisco, California, USA). Serial transverse sections (10 μm thick) were cut from specimens on a cryostat, fixed in acetone at 4°C for five minutes, and then exposed to 0.03% H_2O_2/methanol for 10 minutes at room temperature. Sections were then preincubated in phosphate buffered saline (PBS) supplemented with 10% normal goat serum for three hours before incubating overnight with

Abbreviations: BBB, blood–brain barrier; BMEC, brain microvascular endothelial cell; BNB, blood–nerve barrier; CNS, central nervous system; CIDP, chronic inflammatory demyelinating polyneuropathy; PBS, phosphate buffered saline; PNS, peripheral nervous system; TJ, tight junction; VEGF, vascular endothelial growth factor

Table 1 Clinical features and immunostaining results in 10 patients with chronic inflammatory demyelinating polyradiculoneuropathy

Patient	Age/sex	Biopsy (months)	CSF protein (mg/l)	Symptoms	Course	Nerve conduction findings	Antiganglioside antibodies	Anti-C5+ microvessels (%)	ZO-1+ vessels positive at endothelial cell interfaces (%)
1	13 M	10	1170	Mo>Se	Progr	D	–	27.4	57.1
2	24 M	6	960	Mo=Se	Progr	D	–	70.9	11.1
3	31 M	3	1670	Mo=Se	Progr	D	–	38.4	40.0
4	32 F	4	880	Mo=Se	Progr	D+A	GM1 (IgM)	69.4	78.1
5	41 M	5	680	Mo=Se	RR	D	GM1, GD1b, SGPG	72.6	85.7
6	55 F	2	1140	Mo=Se	Progr	D	–	93.2	†
7	58 M	6	1100	Mo<Se	Progr	D	SGLPG	52.2	41.1
8	62 M	3	1610	Mo<Se	RR	D	–	94.5	13.3
9	63 M	72+	9520	Mo=Se	Progr	D	–	63.2	33.3
10	64 M	2	1850	Mo=Se	RR	D	SGLPG	24.3	55.6

*Percentage of ZO-1 immunoreactive endoneurial vessels showing immunoreactivity at endothelial cell interfaces; †ZO-1 immunoreactivity was too faint for the percentage of vessels showing immunoreactivity at endothelial cell interfaces to be evaluated.
Age, age at the time of biopsy; Biopsy, months from disease onset to biopsy; CSF, cerebrospinal fluid; C5, claudin-5; Mo, motor symptoms; Se, sensory symptoms; Course, clinical course until biopsy; Progr, progressive; RR, relapsing and remitting; D, demyelinating; A, axonal; SGPG, sulfoglucuronosyl paraglycoside; SGLPG, sulfoglucuronosyl lactosaminyl paraglycoside.

primary antibody diluted in PBS. Anti-claudin-5 and anti-occludin antibodies were used at a 1/400 dilution, whereas others were used at a 1/800 dilution. Sections were then rinsed with PBS three times before incubation for one hour with peroxidase conjugated secondary antibody (Nichirei, Tokyo, Japan). The reaction product indicating immunoreactivity in sections was developed with diaminobenzidine.

Analysis of claudin-5 positive microvasculature

We analysed endoneurial microvessels less than 30 μm in diameter, which included capillaries and some precapillary arterioles and postcapillary venules. Vessels apparently surrounded by perineurial cell layers were excluded. Because microvessels in the endoneurium were difficult to count accurately in cryostat sections, whether haematoxylin and eosin stained or immunostained using endothelial markers, such as anti-von Willebrand antigen, we first counted these vessels using toluidine blue stained, plastic embedded semithin sections to evaluate the density of endoneurial microvessels/mm^2. The percentage of claudin-5-positive endoneurial microvessels was calculated as the density of anti-claudin-5 immunoreactive microvessels in cryostat sections ×100/overall density of microvessels in toluidine blue stained sections. If the first density exceeded the last, the percentage of claudin-5 immunoreactive microvessels was considered to be 100%. More than 0.5 mm^2 surface area of endoneurial space was evaluated in each specimen.

Analysis of staining pattern by anti-ZO-1 antibody

The percentage of endoneurial microvessels showing ZO-1 immunoreactivity localised at interfaces between adjoining endothelial cells was evaluated. Microvessels showing two or more immunoreactive lines across the endothelial cell layer, or two or more immunoreactive dots located at the luminal side of endothelial cells (fig 1) were judged to have ZO-1 localised at these endothelial cell borders. The percentage of ZO-1 immunoreactive cells with this localisation pattern was calculated as the number of endoneurial microvessels showing linear or punctate ZO-1 immunoreactivity at borders between endothelial cells ×100/overall number of ZO-1 immunoreactive endoneurial microvessels.

RESULTS

Claudin-1 immunoreactivity was evenly present in the perineurial cell layers, with no clear difference in immunoreactivity between the inner and outer layers. No specimen showed staining of endothelial cells (figs 2A and 3). No difference in claudin-1 immunoreactivity was noted between CIDP and the other disease groups (fig 3).

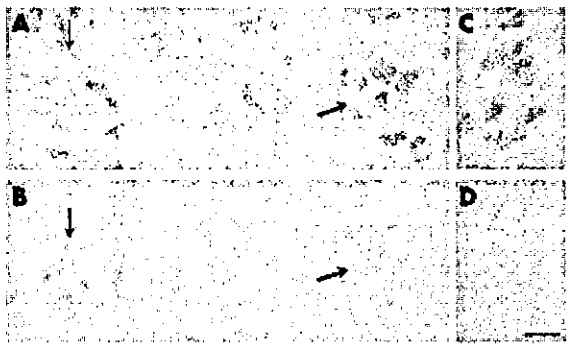

Figure 1 Immunostaining with anti-ZO-1 antibody (B and D) and corresponding serial sections (A and C; haematoxylin and eosin staining) in sural nerves from a 67 year old woman with Churg-Strauss syndrome (A and B) and a 58 year old man with chronic inflammatory demyelinating polyneuropathy (CIPD) (C and D). ZO-1 immunoreactivity in endoneurial microvessels was localised to the interfaces between endothelial cells (arrows), especially at the luminal surface (note the punctate staining of the microvessel on the left in B). In CIDP, endothelial immunoreactivity was diffuse and weak (D); most strong staining localised to the intercellular borders had disappeared. Bar, 10 μm.

The polyclonal anti-occludin antibody stained the perineurial cell layers. In addition, the luminal aspect of some epineurial vessels and a small proportion of endoneurial capillaries adjacent to the perineurium was also immunoreactive (fig 2B). Endoneurial microvessels situated near the centre of the endoneurial space that were not neighbouring perineurial cell layers were not stained. No appreciable difference in immunoreactivity was noted between CIDP and the other disease groups.

Anti-ZO-1 immunoreactivity was localised to the perineurial cell layers, in addition to some endothelial cells lining epineurial vessels and the endoneurium (fig 2C). Although no detectable background staining was seen with the anti-occludin, anti-claudin-1, and anti-claudin-5 antibodies, faint background immunoreactivity was occasionally seen in the endoneurium with the anti-ZO-1 antibody. Therefore, the exact percentage of stained endoneurial microvessels was difficult to determine. Instead, we calculated the percentage of endoneurial microvessels showing immunoreactivity localised to the junctions between endothelial cells (fig 1). In CIDP specimens, the percentage of endoneurial microvessels

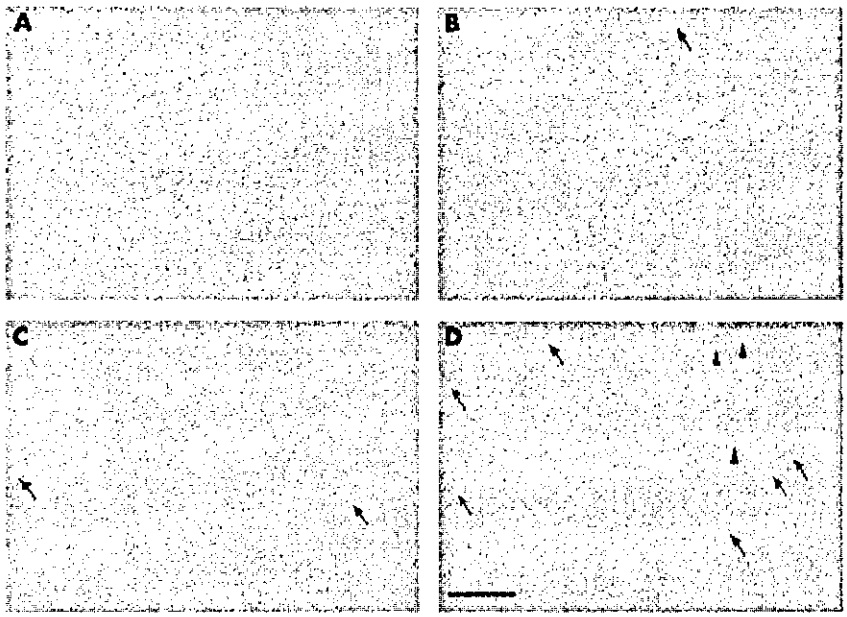

Figure 2 Immunostaining in serial sections obtained from a 64 year old man with Churg-Strauss syndrome with (A) anti-claudin-1, (B) anti-occludin, (C) anti-ZO-1, and (D) anti-claudin-5 antibodies, showing a normal pattern of staining in the human peripheral nervous system. (A) Anti-claudin-1 antibody stained perineurial cell layers exclusively, with no immunoreactivity against endothelial cells. (B) Anti-occludin immunoreactivity was noted in perineurial cell layers. In addition, faint staining was seen in the endothelial cell layers of epineurial vessels (arrow). (C) The anti-ZO-1 antibody reacted faintly with epineurial cell layers and some of the endothelial cells in the endoneurium (arrows). (D) Unlike the other three antibodies, anti-claudin-5 antibody stained endothelial cells exclusively, irrespective of their vessel size or location. Endothelial cells in the epineurium (arrowheads) and in the endoneurium (arrows) were immunoreactive. Bar, 100 μm.

with such anti-ZO-1 immunoreactivity localisation was significantly lower (fig 4).

Claudin-5 was detected exclusively in endothelial cells, irrespective of the location or size of the vessel in the control specimen (figs 2D and 5). Unlike anti-ZO-1 immunostaining, immunoreactivity was not detected at endothelial cell interfaces. In CIDP, the percentage of anti-claudin-5 immunoreactive microvessels in the endoneurium was significantly decreased compared with non-inflammatory neuropathies (figs 5 and 6). No apparent correlation was noted between the loss of claudin-5 immunoreactivity and endoneurial/subperineurial oedema

DISCUSSION

In inflammatory neuropathies including CIDP, increases in cytokines such as interleukin 1β,[11 12] tumour necrosis factor α,[11 12] and vascular endothelial growth factor (VEGF)[13] are believed to contribute to pathogenesis through modulation of the BNB. Among these, VEGF acts as a particularly potent disrupter of the BNB in various inflammatory neuropathies.[14] This increase in vascular permeability occurs through the binding of VEGF to its tyrosine kinase-type receptors, flt-1 and flt-k,[15] resulting in a decrease in TJ proteins, including occludin and vascular endothelial cadherin, and subsequent disorganisation of interendothelial cell junctions.[16] Thus, study of the molecular dynamics of TJ proteins in human inflammatory neuropathies may improve understanding of BNB derangement and prompt the development of new therapeutic approaches in these disorders. Recent experiments have indicated that the establishment of TJ strands depends on claudin family proteins. For example, the transfection of claudin-1 and claudin-2 into fibroblasts induces the formation of TJ strands.[17] Claudin-11 knockout

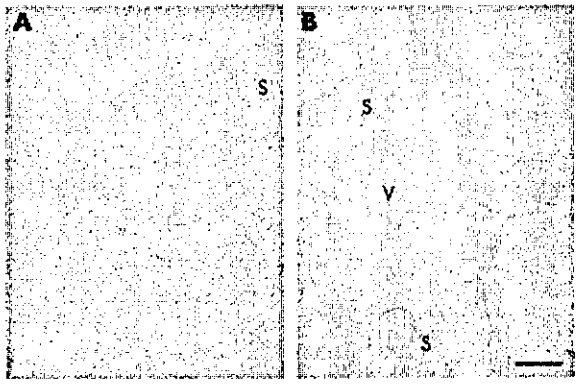

Figure 3 Claudin-1 immunostaining in a control specimen from (A) a 67 year old woman with Churg-Strauss syndrome (CSS) and (B) a specimen from a 55 year old woman with chronic inflammatory demyelinating polyneuropathy (CIDP). Claudin-1 immunoreactivity was detected exclusively in the perineurial cell layers, and no endothelial staining was noted in the endoneurium or epineurium (V). Sections from CIDP and CSS were stained equally. V designates a medium sized epineurial vessel and S designates small sized nerve fascicles. Bar, 100 μm.

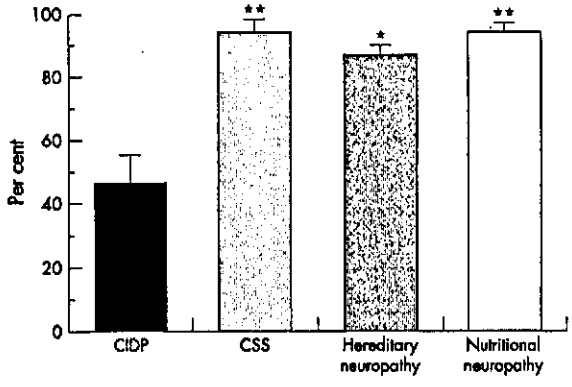

Figure 4 Mean percentage of all ZO-1 immunoreactive endoneurial microvessels showing localisation of ZO-1 immunoreactivity to the interfaces between endothelial cells in specimens with chronic inflammatory demyelinating polyneuropathy (CIDP) (mean, 46.1%; SEM, 8.6%), Churg-Strauss syndrome (CSS) (mean, 93.8%; SEM, 3.7%), hereditary neuropathy (mean, 86.3%; SEM, 3.1%), and nutritional neuropathy (mean, 94.0%; SEM, 2.1%). CIDP specimens showed a significantly lower percentage of endoneurial microvessels with this localisation of ZO-1 immunoreactivity. *$p < 0.005$ v CIDP; **$p < 0.002$ v CIDP. Bars indicate SEM.

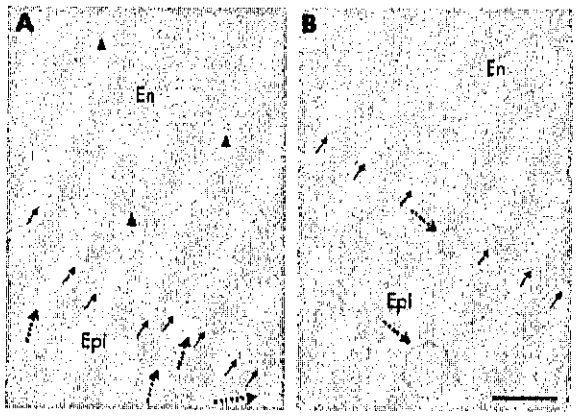

Figure 5 Claudin-5 immunostaining in (A) a control specimen from a 67 year old woman with Churg-Strauss syndrome and (B) a 31 year old man with chronic inflammatory demyelinating polyneuropathy (CIDP). The small arrows indicate the outer aspect of the endoneurium. In the control (A), claudin-5 immunoreactivity was detected exclusively in endothelial cells, irrespective of vessel size or location. Dotted arrows indicate immunoreactive microvessels in the epineurium and arrowheads indicate those in the endoneurium. In CIDP (B), although medium sized vessels in the epineurium were well stained (dotted arrows), immunoreactivity in the endoneurium was severely decreased. En, endoneurium; Epi, epineurium. Bar, 50 μm.

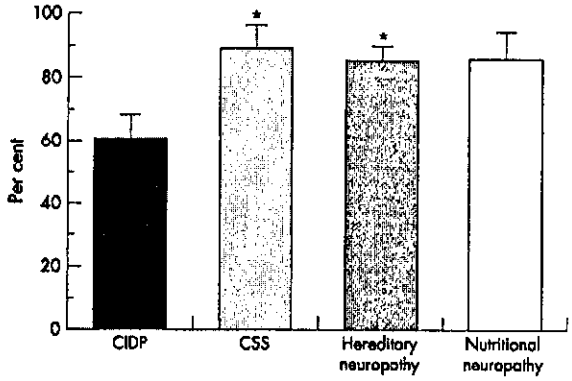

Figure 6 Mean percentage of endoneurial microvessels immunoreactive with anti-claudin-5 antibody in specimens with chronic inflammatory demyelinating polyneuropathy (CIDP) (mean, 60.6%; SEM, 7.8%), Churg-Strauss syndrome (CSS) (mean, 89.5%; SEM, 6.5%), hereditary neuropathy (mean, 85.6%; SEM, 4.0%), and nutritional neuropathy (mean, 86.2%; SEM, 8.4%). Staining with the anti-claudin-5 antibody in the endothelial cells of the endoneurium was significantly decreased in CIDP specimens compared with CSS and hereditary neuropathy. *$p < 0.05$ *v* CIDP. Bars indicate SEM.

mice show absence of TJ strands in the myelin sheets of oligodendrocytes and Sertoli cells,[18] whereas claudin-16 knockout mice demonstrate abnormal paracellular passage of Mg^{2+} ions.[19] Accordingly, we first focused on the expression of two claudin family members, claudin-1 and claudin-5, in sural nerve biopsy specimens.

Claudin-5, which localises exclusively to borders between adjacent endothelial cells,[20] is considered to be important in the control of vascular permeability. Recently Nitta *et al* reported the selective leakage of small molecules (< 800 Da) across the BBB in claudin-5 deficient mice.[21] This suggests that claudin-5, although ubiquitously present in all endothelial cells, plays a special role in the barrier mechanism of the nervous system by preventing the entrance of such small molecules. In their article, no information was given about the BNB, but free entrance of small molecules through claudin-5 deficient endothelial junctions may also occur in the PNS. Hence, the loss of claudin-5 in the endoneurial microvessels in patients with CIDP may enhance the leakage of small molecules into the endoneurial space, and may result in changes to the endoneurial constituents that are unfavourable to Schwann cells and axons, eventually leading to the worsening of neuropathy. However, we found no correlation between the loss of claudin-5 immunoreactivity and the presence of endoneurial/subperineurial oedema; in addition, $Cld5^{-/-}$ mice do not show vasogenic oedema. This might be explained by the fact that even in claudin-5 deficiency most serum proteins (molecular mass > 800 Da) cannot extravasate.[21] Endoneurial/subperineurial oedema, a relatively common finding in sural nerve specimens from patients with CIDP,[22] may be elicited by factors other than a decrease in claudin-5 expression.

The disagreement between our BNB findings and previous observations concerning the BBB[20] is somewhat difficult to account for; one explanation is that downregulation of claudin-5 in endoneurial microvessels in CIDP might be a BNB specific phenomenon, reflecting differences in anatomic structure and cell populations (for example, the absence of astrocytes). Of 10 CIDP specimens, two (from patients 6 and 8) showed normal anti-claudin-5 immunoreactivity in endoneurial microvessels; this could reflect a non-uniform or multifocal, rather than diffuse, distribution of demyelinative foci in this disorder.[23] However, these two specimens showed extremely faint ZO-1 immunoreactivity (patient 6) or a very low percentage of endoneurial vessels showing ZO-1 immunoreactivity at intercellular interfaces (patient 8). Thus, changes of expression of claudin-5 and ZO-1 are not always in parallel with CIDP, suggesting functional differences between these two TJ proteins.

Although claudin-1 is the only member of the claudin family (except for claudin-5) that might be expected to be present in endothelial cells, we found no staining for claudin-1 in the endothelial cells of the PNS. Instead, the anti-claudin-1 antibody exclusively stained the perineurial cell layers. Because the BNB includes the endoneurial microvasculature and the innermost layer of the perineurium,[24 25] anti-claudin-1 immunoreactivity might be taken to represent the latter. However, the uniform staining of all perineurial cell layers that was seen does not correspond well to the site of the BNB. In addition, claudin-1 immunoreactivity was almost as abundant in CIDP as in control specimens. Therefore, claudin-1 is not a marker of BNB integrity.

In our present study, we detected occludin immunoreactivity in endothelial cells of some epineurial vessels, a small percentage of endoneurial capillaries adjoining perineurial cell layers, and perineurial cells. However, we saw no appreciable differences in occludin immunoreactivity in the perineurial cell layer and in the endothelial cells between the various disorders. Occludin may not be essential for TJ formation,[4] but it has been reported to be abundant in relation to the endothelial cells of the brain, although it is undetectable in non-neural tissues.[5 26] VEGF treatment of brain microvascular endothelial cell (BMEC) monolayer cultures decreased detectable occludin and disrupted its continuous pericellular distribution.[27] Although these reports suggest that occludin may play some part in the maintenance of BNB integrity, our results indicated that occludin does not change appreciably in inflammatory neuropathies.

ZO-1, a 220 kDa TJ phosphoprotein, is a member of the membrane associated guanylate kinases localised to intercellular contacts.[28] ZO-1 binds various proteins, including claudins and occludin, and may act as a molecular scaffold bringing these TJ constituents together.[29] Therefore, ZO-1 is expected to be a key molecule in the control of BNB integrity,

despite some previous conclusions that the expression and localisation of ZO-1 do not correlate with the physiological efficiency of paracellular barrier function.[30] In CIDP specimens, we found no significant decrease in ZO-1 immunoreactive endoneurial microvessels, although we noted a change in the staining pattern. This corresponds well with a recent observation that VEGF, known to open the BBB and BNB,[14] caused a loss of ZO-1 from endothelial cell junctions and changed the staining pattern at the cell boundary without decreasing ZO-1 content in cultured bovine BMEC.[27] We suspect that the change in the localisation of ZO-1 in CIDP specimens was an effect of various cytokines, including VEGF, that are upregulated in inflammatory neuropathies such as CIDP. The contribution of other inflammatory cytokines in addition to VEGF requires future investigation.

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Competing interest: none declared

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