damage only in the presence of complement in the present study.

The interaction of endothelial VCAM-1 with the VLA-4 expressed on leukocytes plays a key role in the transmigration of lymphocytes across the BBB, and is implicated in both the capture and strong adhesion of leukocytes to CNS microvessels.[34 35] Our results demonstrated that the whole sera and purified IgG obtained from NMOSD patients increase the expression level of VCAM-1. We thus speculate that anti-AQP4 and/or anti-BMECs antibodies in the NMOSD sera are responsible for the upregulation of VCAM-1 in BMECs. A 50% or 75% reduction in the amount of anti-AQP4 antibodies in the sera obtained from NMOSD patients did not influence the ability of the sera to increase the VCAM-1 expression in the BMECs. These results support our hypothesis that anti-BMEC antibodies, other than anti-AQP4 antibodies, may contribute to the upregulation of VCAM-1 in the BBB, thereby causing the extravasation of inflammatory cells into the CNS parenchyma. However, anti-AQP4 antibodies remain a candidate causative factor involved in the disruption of the BBB, as anti-AQP4 antibodies could not be eliminated completely from NMO sera using the methods employed in this study, although they may only cause damage to the BBB in the presence of human complement. Natalizumab works primarily by blocking the interaction of α4-integrins with the VCAM-1 expressed on the endothelial cell surface, thereby preventing the transmigration of lymphocytes across the BBB.[36 37] Our results suggest that the administration of natalizumab may be clinically effective against NMO. However, several authors have reported that treatment with natalizumab may exacerbate the disease and/or is ineffective in cases of NMO because the drug does not affect the entry of neutrophils into the CNS, which plays an important role in the development of NMO, and because it induces an increase in the number of circulating CD138 plasma cells and mature B cells via the redistribution of lymphocyte subsets in the periphery, causing an increase in the circulating anti-AQP4 antibody level.[38–40] Novel approaches for inhibiting the upregulation of VCAM-1 in the BBB without increasing the secretion of circulating anti-AQP4 antibodies are needed to develop novel therapies for NMO.

In conclusion, our study demonstrated that humoral factors other than IgG that are present only in the sera of patients during the acute phase of NMO may decrease the barrier function of the BBB by increasing the autocrine secretion of MMP-2/9 by BMECs. Additionally, IgG, other than anti-AQP4 antibodies, obtained from NMO sera may upregulate the VCAM-1 expression in the BBB. These findings suggest that key molecules trigger the BBB breakdown observed in the pathogenesis of NMO. Increasing understanding of the molecular mechanism(s) responsible for BBB breakdown in patients with NMO may lead to the development of improved therapeutic strategies for treating this severe and currently treatment-refractory disease.

Contributors AT performed the experiments, analysed and interpreted the data and wrote the manuscript. FS performed the experiments, analysed the data, evaluated the data and edited the manuscript. YS, MF, TT, MA, HH and MA performed the experiments and analysed the data. MK evaluated the data and edited the manuscript. TK conducted and supervised the study, evaluated the data and wrote the manuscript.

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Competing interests None.

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REFERENCES

1 Jarius S, Wildemann B. The history of neuromyelitis optica. *J Neuroinflammation* 2013;10:8.
2 Lennon VA, Wingerchuk DM, Kryzer TJ, *et al*. A serum autoantibody marker of neuromyelitis optica: distinction from multiple sclerosis. *Lancet* 2004;264:2106–12.
3 Hinson SR, Pittock SJ, Lucchinetti CF, *et al*. Pathogenic potential of IgG binding to water channel extracellular domain in neuromyelitis optica. *Neurology* 2007;69:2221–31.
4 Jarius S, Wildemann B. AQP4 antibodies in neuromyelitis optica: diagnostic and pathogenetic relevance. *Nat Rev Neurol* 2010;6:383–92.
5 Misu T, Fujihara K, Kakita A, *et al*. Loss of aquaporin 4 in lesions of neuromyelitis optica: distinction from multiple sclerosis. *Brain* 2007;130:1224–34.
6 Takahashi T, Fujihara K, Nakashima I, *et al*. Anti-aquaporin-4 antibody is involved in the pathogenesis of NMO: a study on antibody titre. *Brain* 2007;130:1235–43.
7 Jacob A, McKeon A, Nakashima I, *et al*. Current concept of neuromyelitis optica (NMO) and NMO spectrum disorders. *J Neurol Neurosurg Psychiatry* 2013;84:922–30.
8 Papadopoulos MC, Verkman AS. Aquaporin 4 and neuromyelitis optica. *Lancet Neurol* 2012;11:535–44.
9 Sharma R, Fischer MT, Bauer J, *et al*. Inflammation induced by innate immunity in the central nervous system leads to primary astrocyte dysfunction followed by demyelination. *Acta Neuropathol* 2010;120:223–36.
10 Bradl M, Misu T, Takahashi T, *et al*. Neuromyelitis optica: pathogenicity of patient immunoglobulin in vivo. *Ann Neurol* 2009;66:630–43.
11 Jarius S, Aboul-Enein F, Waters P, et *al*. Antibody to aquaporin-4 in the long-term course of neuromyelitis optica. *Brain* 2008;131:3072–80.
12 Kim W, Lee JE, Li XF, *et al*. Quantitative measurement of anti-aquaporin-4 antibodies by enzyme-linked immunosorbent assay using purified recombinant human aquaporin-4. *Mult Scler* 2012;18:578–86.
13 Uzawa A, Mori M, Masuda S, *et al*. CSF high-mobility group box 1 is associated with intrathecal inflammation and astrocytic damage in neuromyelitis optica. *J Neurol Neurosurg Psychiatry* 2013;84:517–22.
14 Vincent T, Saikali P, Cayrol R, *et al*. Functional consequences of neuromyelitis optica-IgG astrocyte interactions on blood-brain barrier permeability and granulocyte recruitment. *J Immunol* 2008;181:5730–37.
15 Shimizu F, Sano Y, Takahashi T, *et al*. Sera from neuromyelitis optica patients disrupt the blood-brain barrier. *J Neurol Neurosurg Psychiatry* 2012;83:288–97.
16 Hosokawa T, Nakajima H, Doi Y, *et al*. Increased serum matrix metalloproteinase-9 in neuromyelitis optica: implication of disruption of blood-brain barrier. *J Neuroimmunol* 2011;236:81–6.
17 Uzawa A, Mori M, Masuda S, *et al*. Markedly elevated soluble intercellular adhesion molecule 1, soluble vascular cell adhesion molecule 1 levels, and blood-brain barrier breakdown in neuromyelitis optica. *Arch Neurol* 2011;68:913–17.
18 Leppert D, Ford J, Stabler G, *et al*. Matrix metalloproteinase-9 (gelatinase B) is selectively elevated in CSF during relapses and stable phases of multiple sclerosis. *Brain* 1998;121:2327–34.
19 Murphy G, Knäuper V. Relating matrix metalloproteinase structure to function: why the "hemopexin" domain? *Matrix Biol* 1997;15:511–18.
20 Wingerchuk DM, Lennon VA, Pittock SJ, *et al*. Revised diagnostic criteria for neuromyelitis optica. *Neurology* 2006;66:1485–9.
21 Wingerchuk DM, Lennon VA, Lucchinetti CF, *et al*. The spectrum of neuromyelitis optica. *Lancet Neurol* 2007;6:805–15.
22 Polman CH, Reingold SC, Banwell B, *et al*. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. *Ann Neurol* 2011;69:292–302.
23 Sano Y, Shimizu F, Abe M, *et al*. Establishment of a new conditionally immortalized human brain microvascular endothelial cell line retaining an in vivo blood-brain barrier function. *J Cell Physiol* 2010;225:519–28.
24 Haruki H, Sano Y, Shimizu F, *et al*. NMO sera down-regulate AQP4 in human astrocyte and induce cytotoxicity independent of complement. *J Neurol Sci* 2013;331:136–44.
25 Abe M, Sano Y, Maeda T, *et al*. Establishment and characterization of human peripheral nerve microvascular endothelial cell lines: a new in vitro blood-nerve barrier (BNB) model. *Cell Struct Funct* 2012;37:89–100.
26 Saito K, Shimizu F, Koga M, *et al*. Blood-brain barrier destruction determines Fisher/Bickerstaff clinical phenotypes: an in vitro study. *J Neurol Neurosurg Psychiatry* 2013;84:756–65.
27 Shimizu F, Sano Y, Tominaga O, *et al*. Advanced glycation end-products disrupt the blood-brain barrier by stimulating the release of transforming growth factor-β by pericytes and vascular endothelial growth factor and matrix metalloproteinase-2 by endothelial cells in vitro. *Neurobiol Aging* 2013;34:1902–12.

28 Jarius S, Franciotta D, Paul F, *et al*. Cerebrospinal fluid antibodies to aquaporin-4 in neuromyelitis optica and related disorders: frequency, origin, and diagnostic relevance. *J Neuroinflammation* 2010;7:52.
29 Kinoshita M, Nakatsuji Y, Kimura T, *et al*. Anti-aquaporin-4 antibody induces astrocytic cytotoxicity in the absence of CNS antigen-specific T cells. *Biochem Biophys Res Commun* 2010;394:205–10.
30 Saadoun S, Waters P, Bell BA, *et al*. Intra-cerebral injection of neuromyelitis optica immunoglobulin G and human complement produces neuromyelitis optica lesions in mice. *Brain* 2010;133:349–61.
31 Jarius S, Paul F, Franciotta D, *et al*. Cerebrospinal fluid findings in aquaporin-4 antibody positive neuromyelitis optica: results from 211 lumbar punctures. *J Neurol Sci* 2011;306:82–90.
32 Gijbels K, Galargy RE, Steinman L. Reversal of experimental autoimmune encephalomyelitis with a hydroxamate inhibitor of matrix metalloproteinases. *J Clin Invest* 1994;94:2177–82.
33 Augé F, Hornebeck W, Decarme M, *et al*. Improved gelatinase a selectivity by novel zinc binding groups containing galardin derivatives. *Bioorg Med Chem Lett* 2003;13:1783–6.
34 Engelhardt B. T cell migration into the central nervous system during health and disease: different molecular keys allow access to different central nervous system compartments. *Clin Exp Neuroimmunol* 2010;1:79–93.
35 Takeshita Y, Ransohoff RM. Inflammatory cell trafficking across the blood-brain barrier: chemokine regulation and in vitro models. *Immunol Rev* 2012;248: 228–39.
36 Engelhardt B, Kappos L. Natalizumab: targeting alpha4-integrins in multiple sclerosis. *Neurodegener Dis* 2008;5:16–22.
37 Miller DH, Soon D, Fernando KT, *et al*. AFFIRM Investigators. MRI outcomes in a placebo-controlled trial of natalizumab in relapsing MS. *Neurology* 2007;68:1390–401.
38 Barnett MH, Prineas JW, Buckland ME, *et al*. Massive astrocyte destruction in neuromyelitis optica despite natalizumab therapy. *Mult Scler* 2012;18:108–12.
39 Kleiter I, Hellwig K, Berthele A, *et al*. Failure of natalizumab to prevent relapses in neuromyelitis optica. *Arch Neurol* 2012;69:239–45.
40 Jacob A, Hutchinson M, Elsone L, *et al*. Does natalizumab therapy worsen neuromyelitis optica? *Neurology* 2012;79:1065–6.

RESEARCH PAPER

Sera from patients with multifocal motor neuropathy disrupt the blood-nerve barrier

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ABSTRACT

Objective In multifocal motor neuropathy (MMN), the destruction of the blood-nerve barrier (BNB) has been considered to be the key step in the disease process. The purpose of the present study was to ascertain whether sera from patients with MMN can open the BNB, and which component of patient sera is the most important for this disruption.

Methods We evaluated the effects of sera from patients with MMN, patients with amyotrophic lateral sclerosis, and control subjects on the expression of tight junction proteins and vascular cell adhesion molecule-1 (VCAM-1), and on the transendothelial electrical resistance (TEER) in human peripheral nerve microvascular endothelial cells (PnMECs).

Results The sera from patients with MMN decreased the claudin-5 protein expression and the TEER in PnMECs. However, this effect was reversed after application of an anti-vascular endothelial growth factor (anti-VEGF) neutralising antibody. The VEGF secreted by PnMECs was significantly increased after exposure to the sera from patients with MMN. The sera from patients with MMN also increased the VCAM-1 protein expression by upregulating the nuclear factor kappa-B (NF-κB) signalling. The immunoglobulin G purified from MMN sera decreased the expression of claudin-5 and increased the VCAM-1 expression in PnMECs.

Conclusions The sera from MMN patients may disrupt the BNB function via the autocrine secretion of VEGF in PnMECs, or the exposure to autoantibodies against PnMECs that are contained in the MMN sera. Autoantibodies against PnMECs in MMN sera may activate the BNB by upregulating the VCAM-1 expression, thereby allowing for the entry of a large number of circulating inflammatory cells into the peripheral nervous system.

INTRODUCTION

Multifocal motor neuropathy (MMN) is an acquired neuropathy characterised by chronic or stepwise progressive asymmetrical limb weakness without sensory defects.[1 2] The etiopathogenesis of MMN is not well known, but there is some evidence that the disease has an immunological basis, because immunological therapies including high-dose intravenous immunoglobulins (IVIg) show therapeutic effects, although corticosteroids and plasma exchange are largely ineffective.[3–8] Anti-GM1 IgM antibodies can be found in some patients with MMN,[2 9] but it is unclear whether these antibodies are pathogenic. However, some reports support the hypothesis that autoantibodies that bind to gangliosides activate the classical complement system pathway and induce nerve injury by the incorporation of the complement membrane attack complex (C5b-9) in peripheral motor nerves.[10–12]

The blood-nerve barrier (BNB) protects the nerve fibres in the PNS from systemic inflammatory reactions and immune responses.[13 14] Several lines of evidence have demonstrated that the disruption of the BNB, causing the leakage of macromolecules like immunoglobulin and cytokines, is a key step in the disease process of chronic inflammatory demyelinating polyneuropathy (CIDP).[15] A few reports about the pathological findings in the motor nerves of a patient with MMN suggested that the disruption of the BNB may occur during the disease process of MMN.[16–18] However, it has not been adequately explained whether the sera from patients with MMN can disrupt the BNB, and which component of the patients' sera is the most critical for the dysregulation of the BNB.

The purpose of the current study was to demonstrate the effects of sera from patients with MMN on the impairment of the BNB function, and to clarify the roles of humoral factors, especially antibodies against the human BNB-composing endothelial cells, in the destruction of the BNB.

MATERIALS AND METHODS

Sera

This study was approved by the review boards of Tokushima University and Yamaguchi University following the principles of the Declaration of Helsinki. All patients consented to participate in this study. The acute-phase sera were collected from 11 patients with MMN who were diagnosed at Tokushima University Hospital or Yamaguchi University Hospital (table 1). All 11 patients met the clinical criteria for possible MMN based on the 2010 EFNS/PNS guideline[19] and had an objective clinical improvement following IVIg treatment. Three of the 11 patients with MMN (patient nos. 4, 6, 7) were positive for anti-GM1 IgM antibodies (table 1). The sera from nine patients with definite amyotrophic lateral sclerosis (ALS) diagnosed by the El Escorial criteria[20] were also used in this study as disease controls. The sera from 10 healthy individuals served as normal controls. Blood samples were taken before treatment and stored at −80°C until use. All sera were incubated at 56°C for 30 min just prior to use.

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Table 1 Clinical profiles and nerve conduction data of patients with MMN

	Patient nos.										
	1	2	3	4	5	6	7	8	9	10	11
Age at onset (year)/sex	53/M	36/F	20/M	46/M	57/M	57/M	32/F	51/F	63/M	16/M	16/M
Duration (year)	3	5	4	17	1	4	1	10	1	1	2
Site of onset	R/UL	R/UL	R/UL	R/LL	L/UL	L/UL	R/UL	L/UL	L/UL	R/UL	R/UL
Currently affected nerves	RL/Med RL/Uln RL/Rad	R/Mus R/Med R/Uln R/Tib	RL/Uln R/Rad R/Per R/Tib	L/Mus L/Uln L/Rad R/Per R/Tib	L/Mus RL/Uln RL/Rad L/Per	L/Med L/Rad	R/Med R/Uln R/Rad	L/Med RL/Uln R/Rad	L/Med L/Uln L/Rad	R/Uln R/Rad	R/Med R/Uln
Asymmetrically?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Tendon reflexes in the affected limbs	Absent	Absent	Absent	Decreased	Decreased	Decreased	Normal	Normal	Normal	Normal	Normal
Response to IVIg?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
CSF protein (mg/dL)	39	36	21	46	66	NA	NA	NA	NA	NA	42
Positive for serum anti-GM1 IgM antibodies?	No	No	No	Yes	No	Yes	Yes	No	No	No	No
Motor conduction study											
Median nerve											
Distal latency (ms)	5.6	3.7	5.1	3.5	3.8	NA	4.1	4.8	2.8	3.0	3.1
CV (m/s)	64	54.6	31.6	55.0	55.9	NA	58.0	67.0	61.0	58.0	55.0
CMAP amp (mV) (proximal/distal)	0.2/0.4	7.0/9.9	0.2/0.5	3.8/4.2	5.0/5.1	NA	1.2/1.2	1.2/3.1	7.3/7.4	7.3/7.5	6.8/7.0
CMAP area (mVms) (proximal/distal)	0.5/0.8	23.7/25.0	1.1/2.8	10.6/10.8	15.2/15.8	NA	2.6/2.6	3.8/10.4	21.4/21.5	23.9/25.4	18.5/20.1
Duration increase (%) (proximal/distal)	28%	3%	22%	6%	2%	NA	3%	21%	8%	4%	2%
F wave lat (ms)	NA	25.3	35.7	27.4	NA	NA	25.2	25.8	25.6	26.0	26.9
Ulnar nerve											
Distal latency (ms)	3.3	2.5	3.2	3.5	3.0	2.9	2.1	2.5	2.9	3.3	2.8
CV (m/s)	53.0	59.5	48.7	34.0	62.2	62.5	66.0	38.0	61.0	79.0	62.0
CMAP amp (mV) (proximal/distal)	5.3/5.6	5.1/5.2	8.7/11.0	2.9/4.1	4.2/4.4	5.6/6.2	7.4/7.9	1.5/2.2	0.6/1.2	5.2/5.8	4.4/4.4
CMAP area (mVms) (proximal/distal)	6.1/6.1	21.6/25.0	17.0/17.0	11.1/15.1	11.1/11.4	19.6/21.8	20.8/22.4	3.2/5.9	13.2/21.4	12.1/13.1	14.6/15.3
Duration increase (%) (proximal/distal)	25%	4%	0%	3%	5%	29%	0%	0%	13%	5%	0%
F wave lat (ms)	29.0	23.1	33.0	28.4	29.9	NA	23.0	28.9	NA	27.7	27.0
Tibial nerve											
Distal latency (ms)	4.6	4.6	6.6	4.9	5.2	NA	NA	3.5	NA	NA	NA
CV (m/s)	40	47.1	28.7	47.0	43.3	NA	NA	44.8	NA	NA	NA
CMAP amp (mV) (proximal/distal)	2.6/5.5	7.9/9.6	1.1/2.1	1.8/2.4	10.2/14.2	NA	NA	7.7/10.7	NA	NA	NA
F wave lat (ms)	56.0	40.3	84.1	54.4	51.0	NA	NA	40.4	NA	NA	NA
Distribution of CB (definite or probable CB)	Med (probable)	None	Med (probable)	None	None	None	None	Med (probable)	Uln (probable)	None	None
Diagnostic categories	Possible	Possible	Possible	Possible	Possible	Possible	Possible	Possible	Possible	Possible	Possible

CB, conduction block; CMAP amp, compound muscle action potential amplitude; CV, conduction velocity; F wave lat, F wave latency; L, left; LL, lower limb; Med, median nerve; MMN, multifocal motor neuropathy; Mus, musculocutaneus nerve; NA, not available; Per, deep peroneal nerve; R, right; Rad, radial nerve; RL, right and left; Tib, tibial nerve; UL, upper limb; Uln, ulnar nerve.

Cell culture and treatment

The immortalised human peripheral nerve microvascular endothelial cells (PnMECs), which were named 'FH-BNB', were generated previously.[14 21] The PnMECs were treated with culture medium containing 10% patient or healthy control sera in a humidified atmosphere of 5% CO_2/air. PnMECs treated with culture medium with 10% fetal bovine serum (FBS; Sigma, St. Louis, Missouri, USA) were used as controls. The transendothelial electrical resistance (TEER) value was measured 24 h later, and the total proteins were obtained the next day.

Reagents

The culture medium for PnMECs was previously described.[21] Polyclonal anti-claudin-5 and anti-occludin antibodies were purchased from Zymed (San Francisco, California, USA). The polyclonal anti-actin and anti-nuclear factor kappa-B (anti-NF-κB) p65 antibodies were obtained from Santa Cruz (Santa Cruz, California, USA). The polyclonal anti-IL-1β, anti-TNF-α, anti-TGF-β, anti-vascular endothelial growth factor (anti-VEGF), anti-IL-6, and anti-vascular cell adhesion molecule-1 (VCAM-1) antibodies were purchased from R&D Systems (Minneapolis,

Minnesota, USA). The broad-spectrum matrix metalloproteinase (MMP) inhibitor, GM6001, was purchased from Chemicom (Temecula, California, USA).

Western blot analysis

The protein samples (10–20 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad), and then were transferred to nitrocellulose membranes (Amersham, Chalfont, UK) as described previously.[21] The membranes were treated with relevant antibodies (dilution 1 : 100) for 2 h as the primary antibodies and then incubated with secondary antibodies (dilution 1 : 2000) for 1 h at room temperature. The membranes were visualised by enhanced chemiluminescence detection (ECL-prime, Amersham, UK). A densitometric analysis was performed using the Quantity One software program (Bio-Rad, Hercules, California, USA).

TEER studies

The TEER values of cell layers were measured with a Millicell electrical resistance apparatus (Endohm-6 and EVOM, World Precision Instruments, Sarasota, Florida, USA) as described previously.[21] The PnMECs were seeded (1×10^6 cells/insert) on the upper compartment and incubated with each type of medium (non-conditioned medium used as a control, conditioned medium contained 10% patient sera) for 24 h.

Permeability studies

The PnMECs were grown to confluence on 24-well tissue culture inserts (0.4 μm pore size, 1.0×10^4 cells/insert) as described previously.[14] About 1 300 μL of the culture medium was added to the lower well, and 500 μL of culture medium containing sodium fluorescein (10 μg/mL) of molecular weight 400 kDa was added to the upper compartment of each insert. After incubation for 15, 30, 45 or 60 min at 37°C, the lower chamber was sampled and the fluorescence was measured using an MX3000P instrument (Stratagene).

Quantitative analysis of VEGF by ELISA

The serum levels of VEGF were determined in triplicate by an ELISA using commercially available kits (R&D Systems, Minneapolis, Minnesota, USA). The results were expressed as picograms of VEGF per millilitre (pg/mL), based on the standards provided with the available kits.

Treatment with neutralising antibodies

The sera from patients with MMN were pretreated with either a neutralising antibody (2.0 μg/mL) against IL-1β, TNF-α, TGF-β, IL-6, or VEGF or normal rabbit IgG (control Ab) for 6 h at 4°C. PnMECs were cultured with the sera from three patients with MMN containing each neutralising antibody at 37°C.

Treatment with an MMP inhibitor or NF-κB inhibitor

A broad-spectrum MMP inhibitor, GM6001 (Chemicom, Temecula, California, USA), or NF-κB activation inhibitor (Calbiochem, Darmstadt, Germany) was prepared for the inhibition study. The sera from patients with MMN were pretreated with 25 μM of GM6001 or 150 nM of the NF-κB inhibitor for 12 h at 37°C. PnMECs were cultured with the sera from each of three patients with MMN with GM6001 or the NF-κB inhibitor.

IgG purification from serum

The IgG fractions were obtained from the sera of five patients with anti-GM1 antibody-negative MMN or five healthy individuals by affinity chromatography using a Melon Gel IgG Spin Purification Kit (Thermo Scientific, Rockford, Illinois, USA). Cells were treated with culture medium containing either purified patient or healthy individual IgG (final concentration 400 μg/mL). Cells treated with culture medium containing purified IgG obtained from FBS (Sigma, final concentration 400 μg/mL) were used as controls.

Data analysis

An unpaired, two-tailed Student t test was used to determine the significance of differences between the means of two groups. A p value <0.01 was considered to be statistically significant.

RESULTS

MMN sera decreased the BNB function

Table 1 shows the clinical profiles and nerve conduction data for each of the patients with MMN. We first examined whether the sera from patients with MMN affects the BNB function. The amount of claudin-5 in the PnMECs was significantly decreased after exposure to sera from patients with MMN, whereas it was not affected by the sera from patients with ALS or healthy controls, as determined by a Western blot analysis (figure 1A–D). The amount of occludin protein was not changed after exposure to sera from patients MMN or ALS, or healthy controls (figure 1E). The TEER value of PnMECs was significantly decreased, and the sodium fluorescein (NaF) permeability of PnMECs was significantly increased, after exposure to sera from patients with MMN, although it was not changed by incubation with sera from patients with ALS or healthy controls (figure 1F,G). The presence of anti-GM1 IgM antibodies did not influence either the change of claudin-5 protein amounts or the NaF permeability of the PnMECs (figure 1H,I).

MMN sera increased the amount of VCAM-1 protein through NF-κB signalling in PnMECs

We next analysed whether the sera from patients with MMN affect the expression of adhesion molecule. The amount of vascular cell adhesion molecule-1 (VCAM-1) and NF-κB p65 protein in PnMECs was significantly increased after exposure to sera from patients with MMN or ALS, whereas it was not changed by the sera from healthy controls, as determined by a Western blot analysis (figure 2A–C). To clarify the contribution of NF-κB to the BNB breakdown, we investigated the amount of VCAM-1 and claudin-5 protein, the TEER value and the NaF permeability in PnMECs after MMN sera exposure with or without pretreatment with NF-κB inhibitor. The amount of VCAM-1 protein in PnMECs after exposure to MMN sera after pretreatment with the NF-κB inhibitor was significantly decreased compared with that in cells without pretreatment with the NF-κB inhibitor (figure 2D,E). The amount of claudin-5 protein, the TEER value and the NaF permeability in PnMECs after MMN sera exposure and pretreatment with the NF-κB inhibitor were not changed compared with those in cells without treatment with the NF-κB inhibitor (figure 2F–I).

MMN sera disrupted the BNB through the upregulation of autocrine VEGF in PnMECs

To clarify the contribution of inflammatory cytokines or MMPs to the BNB breakdown in MMN, the TNF-α, IL-1β, IL-6, TGF-β or VEGF activities were neutralised using the corresponding neutralising antibodies or MMPs were inhibited by the broad-spectrum MMP inhibitor, GM6001 (figure 3A–F). The amount of claudin-5 protein in PnMECs was significantly increased after exposure to the MMN sera pretreated with the anti-VEGF neutralising antibody, as determined by a Western

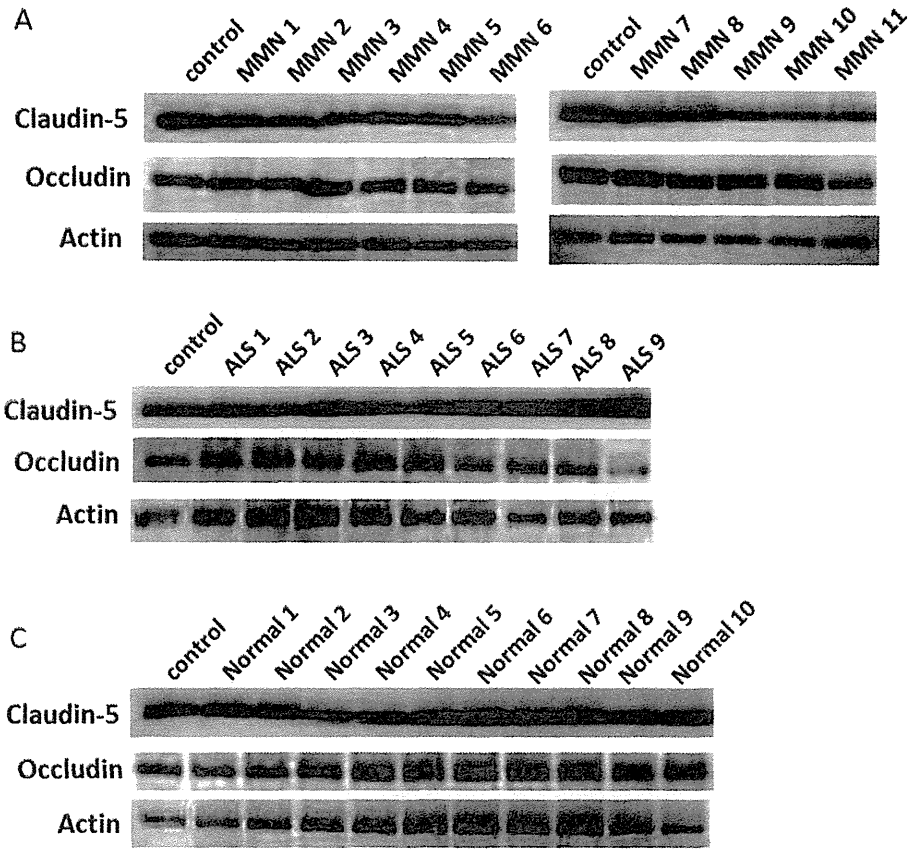
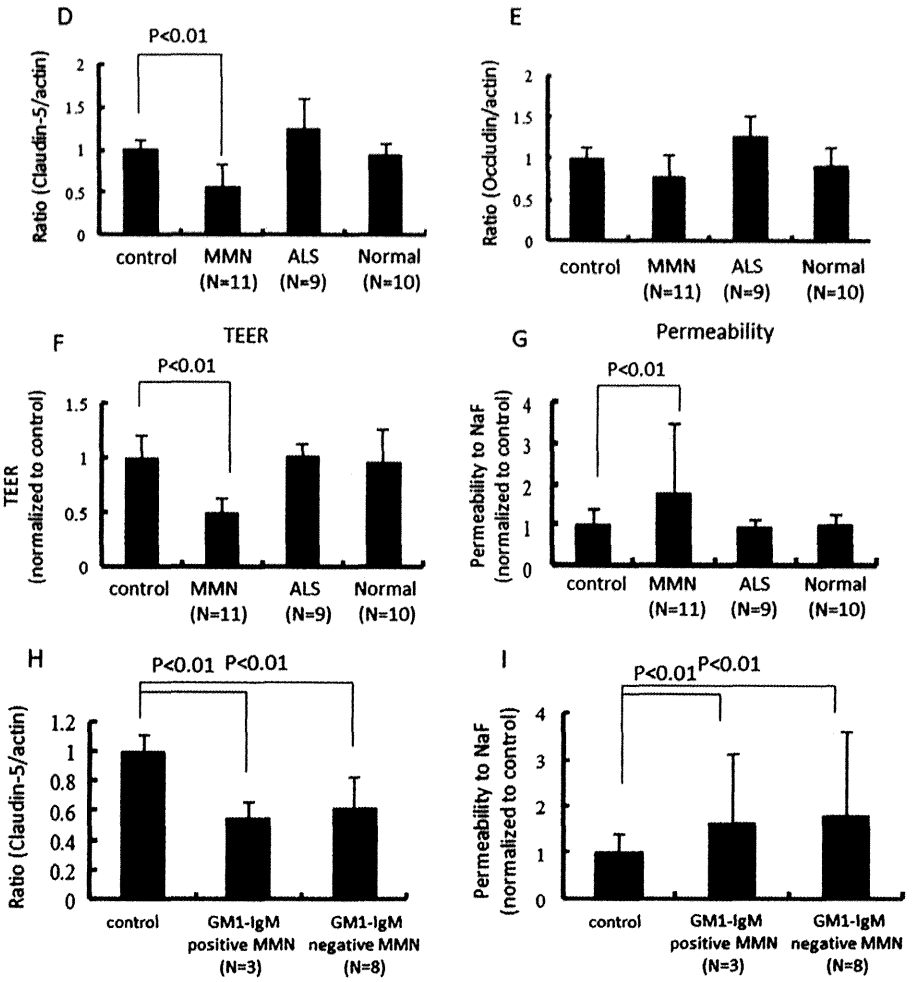


Figure 1 (A–C) The effects of the sera of patient with multifocal motor neuropathy (MMN) on the tight junction proteins in human peripheral nerve microvascular endothelial cells (PnMECs) as determined by a Western blot analysis. The changes of claudin-5 and occludin in PnMECs were determined after exposure to the sera from patients with MMN or amyotrophic lateral sclerosis (ALS), or from healthy controls. (D and E) Each bar graph reflects the combined densitometry data from each independent experiment. The amount of claudin-5 protein in PnMECs was significantly decreased after exposure to the sera from patients with MMN (mean±SEM, n=11, $p<0.01$). The amounts of claudin-5 and occludin were not significantly affected by exposure to the sera from patients with ALS (mean±SEM, n=9) or from healthy controls (mean±SEM, n=10). (F and G) The transendothelial electrical resistance value of PnMECs was significantly decreased (F) and the NaF permeability of PnMECs was significantly increased (G) after exposure to MMN sera, but these were not influenced by exposure to sera from patient with ALS or healthy controls. (H and I) The effect of anti-GM1 IgM antibodies in the sera from patients with MMN on the amount of tight junction proteins and NaF permeability. The amount of claudin-5 protein was decreased, and the NaF permeability was increased after exposure to the sera from patients with MMN with and without anti-GM1 IgM antibodies, compared to that of control, irrespective of the presence of anti-GM1 antibody. Therefore, the presence of anti-GM1 IgM antibodies did not influence the claudin-5 protein amounts (H) or the NaF permeability (I). Control: non-conditioned DMEM containing 20% fetal bovine serum (FBS); MMN: conditioned medium with 10% serum from a patient with MMN diluted with non-conditioned DMEM containing 10% FBS; ALS: conditioned medium with a 10% concentration of serum from a patient with ALS diluted with non-conditioned DMEM containing 10% FBS; Normal: conditioned medium with 10% serum from a healthy control diluted with non-conditioned medium of DMEM containing 10% FBS; GM1-IgM positive MMN, conditioned medium with 10% serum samples of patients with MMN with anti-GM1 IgM antibodies; GM1-IgM negative MMN, conditioned medium with 10% serum samples of patients with MMN without anti-GM1 IgM antibodies.

blot analysis (figure 3F,L), whereas it did not change after preincubation with TNF-α, IL-1β, IL-6 or TGF-β neutralising antibodies or GM6001 (figure 3A–E,G–K). The TEER value of the PnMECs was also significantly increased, and the NaF permeability of PnMECs was significantly decreased after exposure to MMN sera pretreated with the anti-VEGF antibody (figure 3M, N). The serum concentration of VEGF did not significantly differ between the patients with MMN , patients with ALS and healthy controls as groups, although some patients with MMN and ALS had VEGF concentrations higher than the range observed in the healthy controls, as determined using an ELISA method (figure 3O). We thus considered that MMN sera may disrupt the BNB by increasing the autocrine secretion of VEGF in PnMECs. The expression of VEGF in PnMECs was found to be significantly increased after exposure to sera from patients with MMN (figure 3P,R), whereas it did not change after exposure to the sera from healthy controls (figure 3Q,S). The presence of anti-GM1 IgM antibodies did not influence the changes in the amounts of VEGF proteins in the PnMECs (figure 3T).

Purified serum IgG from patients with MMN disrupts the BNB

We next analysed whether autoantibodies against human PnMECs were present in the purified IgG fractions of sera from patients with MMN by a Western blot analysis. Antibodies that bound to PnEMCs were detected in the purified IgG fractions of sera from five patients with MMN patient, which predominantly reacted with one or more antigens of approximately 30, 45, 50, 54, 56 and 70 kDa in PnMEC lysates (figure 4A). Notably, antibodies against the antigens corresponding to 54, 56



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and 70 kDa were specific for patients with MMN and were not seen in the sera from patients with ALS or healthy controls (figure 4A). The lower molecular bands corresponding to 30, 45 and 50 kDa in PnMECs were not specific for patients with MMN, because these bands were commonly detected in ALS patients or healthy controls (figure 4A). We next examined whether the purified serum IgG from patients with MMN, rather than anti-GM1 IgM antibodies, was indeed responsible for the disruption of the BNB. The amount of claudin-5 in PnMECs was significantly decreased after exposure to the purified IgG fractions of sera from anti-GM1 IgM antibody-negative MMN patients, whereas it was not affected by the purified IgG fractions from healthy controls, as determined by a Western blot analysis (figure 4B,C). The amount of VEGF proteins did not change following exposure to the purified serum IgG fractions obtained from the patients with MMN and healthy controls (figure 4B,D). The TEER value of PnMECs was significantly decreased and the NaF permeability of PnMECs was significantly increased after exposure to the purified IgG fraction from anti-GM1 IgM antibody-negative MMN patients, although it was not changed by incubation with the purified IgG fractions from healthy controls (figure 4E,F). In addition, the amount of VCAM-1 and NF-κB p65 in PnMECs was significantly increased after exposure to the purified IgG fraction from anti-GM1 IgM antibody-negative MMN patients, whereas it was not changed by the purified IgG fractions from healthy controls, as determined by a Western blot analysis (figure 4G–I).

DISCUSSION

The etiopathogenesis of MMN has not been clarified. Some evidence suggests that the disease has an immunological basis, primarily due to the occurrence of clinical improvement following the administration of immunological therapy, including high-dose IVIg.[3–7] However, the precise mechanisms and target antigens of this immune response are unknown. An important diagnostic feature is the presence of persisting multifocal partial conduction blocks (CBs) that selectively affected the motor axons in the nerve conduction studies.[3–8] Although the pathological basis of CBs is considered to be focal demyelination, this has rarely been confirmed in MMN by morphological studies, because tissue samples taken from the motor nerves of patients with MMN are extremely rare. Some previous reports on sensory nerve biopsies in patients with MMN have described either normal findings or unspecific changes, consistent with the infrequent sensory impairment in patients with MMN.[22 23] Only a few reports on motor nerve biopsies or autopsies in MMN cases have been published. For example, Kaji *et al*[16] described the myelinated axons and the formation of onion bulbs with endoneurial oedema and perineurial thickening in the medial pectoral nerve biopsy at the site of CB and suggested that

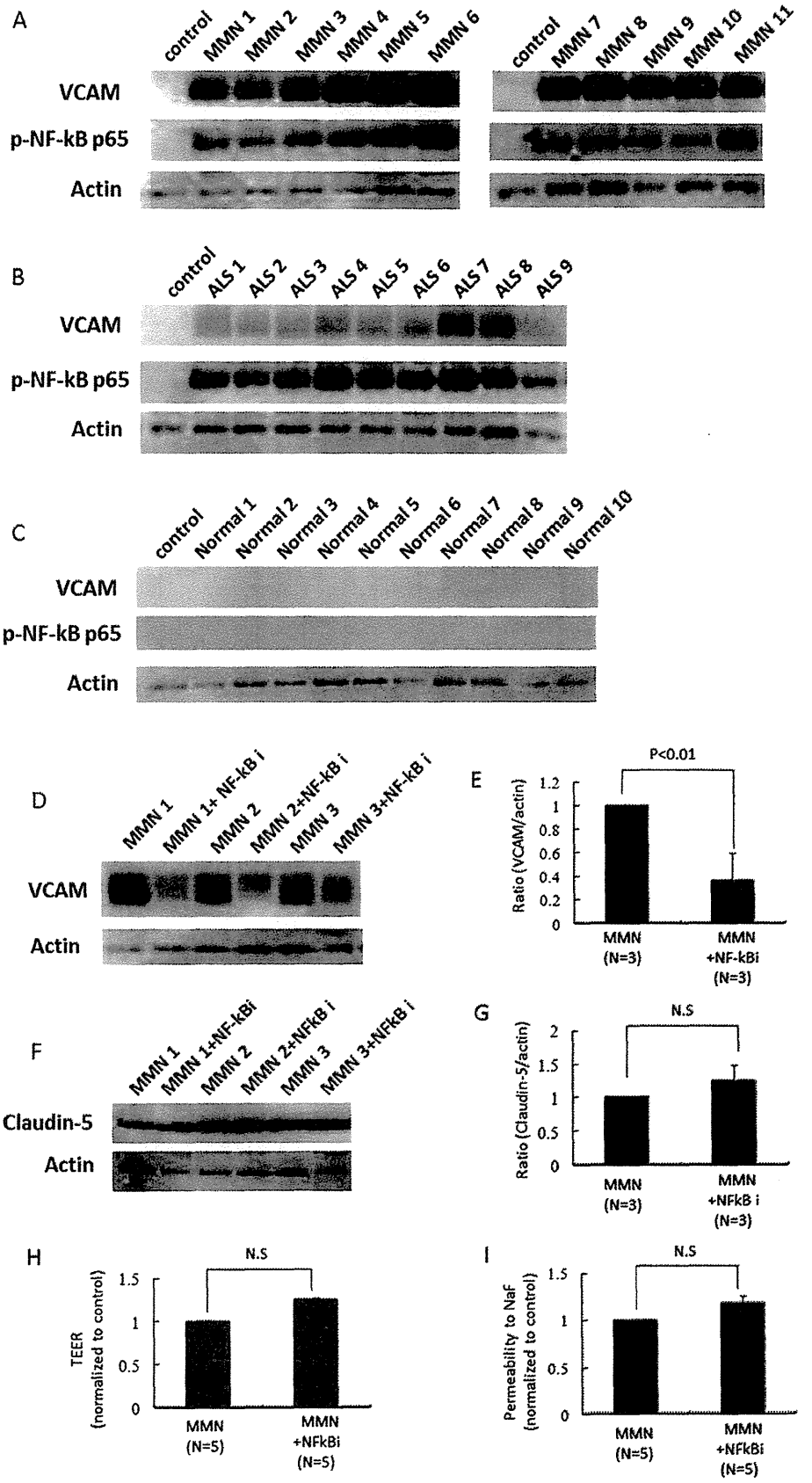


Figure 2 (A–C) The effects of sera on the amount of adhesion molecules in human peripheral nerve microvascular endothelial cells (PnMECs) were determined by a Western blot analysis. The changes in the amount of VCAM-1 protein in PnMECs were determined after exposure to the sera from patients with multifocal motor neuropathy (MMN) (A) or amyotrophic lateral sclerosis (ALS) (B), or from healthy controls (C). (D) The effects of an NF-κB inhibitor on the expression of adhesion molecules in PnMECs after exposure to the sera from a patient with MMN was determined by a Western blot analysis. The amount of VCAM-1 protein in PnMECs after MMN sera exposure in cells pretreated with the NF-κB inhibitor was significantly decreased compared to that of cells without NF-κB inhibitor pretreatment. (E) Each bar graph reflects the combined densitometry data from independent experiments (mean±SEM, n=3, *: $p<0.01$). (F–I) The effects of the NF-κB inhibitor on the amount of claudin-5 protein, the transendothelial electrical resistance (TEER) value and the NaF permeability of PnMECs after exposure to the sera from a patient with MMN. (F) The amount of claudin-5 protein in PnMECs after MMN sera exposure and pretreatment with the NF-κB inhibitor was not changed compared to that of cells without NF-κB inhibitor pretreatment. (G) Each bar graph reflects the combined densitometry data from independent experiments (mean±SEM, n=3, $p<0.01$). (H and I) The TEER value and NaF permeability across PnMECs after MMN sera exposure in cells pretreated with the NF-κB inhibitor were not significantly changed compared to those of cells without NF-κB inhibitor pretreatment. Control: non-conditioned DMEM containing 20% fetal bovine serum (FBS); MMN: conditioned medium with 10% serum from a patient with MMN diluted with non-conditioned DMEM containing 10% FBS; ALS: conditioned medium with a 10% concentration of serum from a patient with ALS diluted with non-conditioned DMEM containing 10% FBS; Normal: conditioned medium with 10% serum from a healthy control diluted with non-conditioned medium of DMEM containing 10% FBS; MMN+NF-κB inhibitor: conditioned medium with 10% MMN sera pretreated with the NF-κB inhibitor.

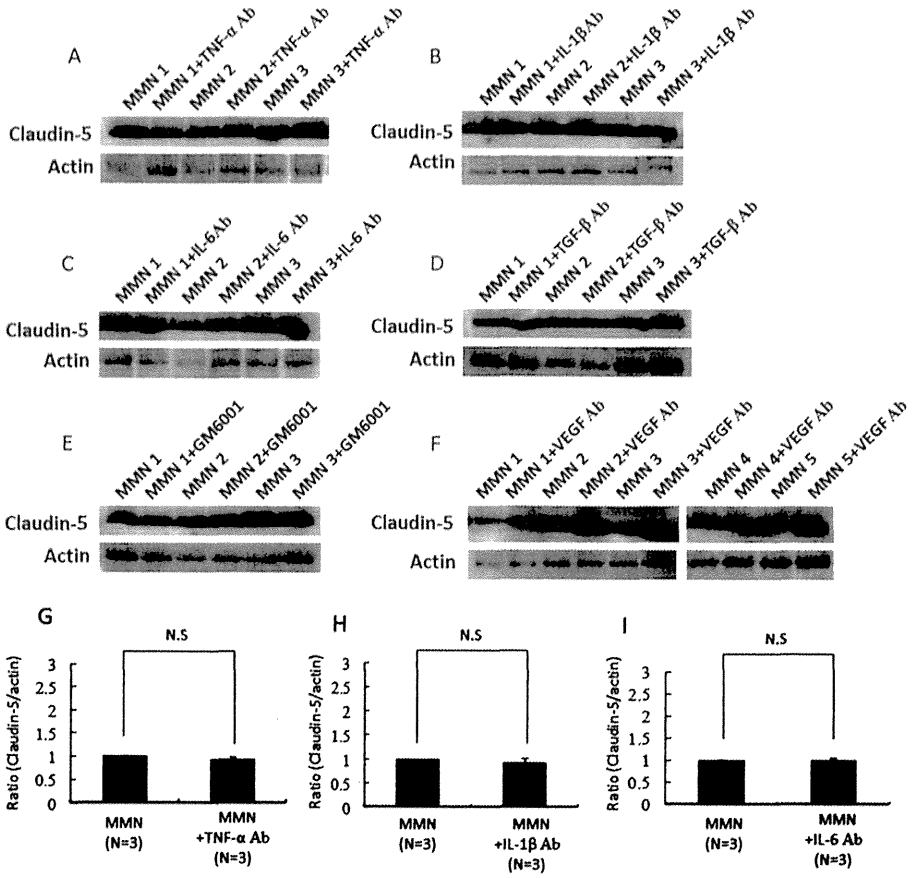
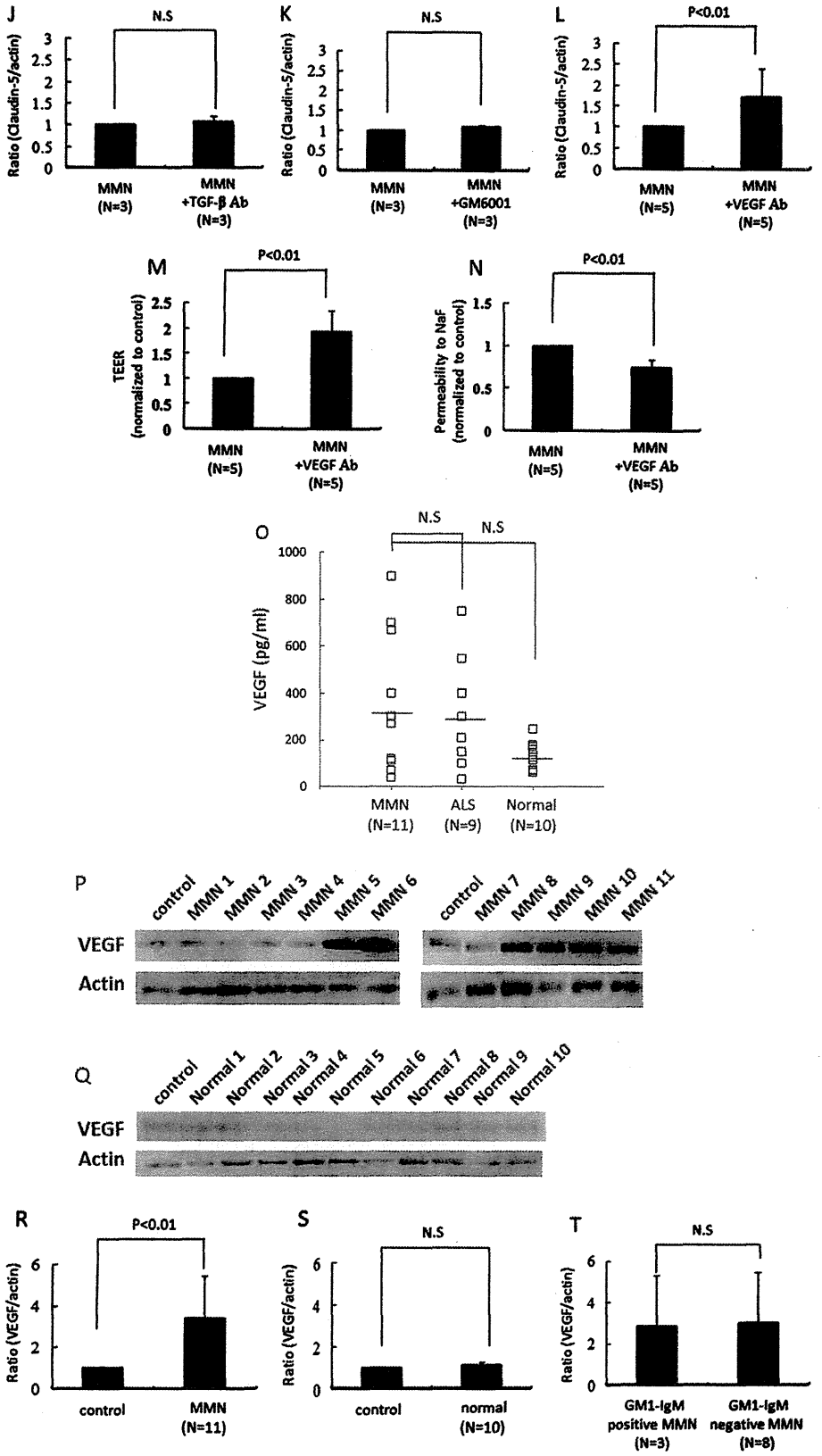


Figure 3 (A–F) The effects of anti-TNF-α, IL-1β, IL-6, TGF-β or vascular endothelial growth factor (VEGF) neutralising antibodies or a matrix metalloproteinase inhibitor on the amount of tight junction proteins in human peripheral nerve microvascular endothelial cells (PnMECs) after exposure to the sera from patients with multifocal motor neuropathy (MMN) was determined by a Western blot analysis. (G–L) Each bar graph reflects the combined densitometry data from each independent experiment (mean±SEM, n=3, *: p<0.01). (F and L) Preincubation with an anti-VEGF neutralising antibody increased the amount of claudin-5 protein in PnMECs (mean±SEM, n=5, *: p<0.01). The transendothelial electrical resistance value of PnMECs significantly increased (M) or the NaF permeability of PnMECs significantly decreased (N) after incubation with the sera from patients with MMN that were pretreated with an anti-VEGF neutralising antibody (mean±SEM, n=5). (O) The serum VEGF concentration was analysed in patients with MMN or amyotrophic lateral sclerosis, or from healthy control subjects. The bars indicate the mean of each group. No significant differences were observed between the three groups. (P–T) The expression of VEGF by PnMECs after exposure to the sera from patients with MMN. The amount of VEGF protein in the PnMECs was significantly increased after exposure to the sera from patients with MMN (P), although it did not change after exposure to the sera from healthy controls (Q). (R and S) Each bar graph reflects the combined densitometry data from each independent experiment (mean±SEM, MMN n=11, healthy control n=10, p<0.01). (T) The presence of anti-GM1 IgM antibodies did not influence the changes in the amounts of VEGF proteins in the PnMECs. MMN: conditioned medium with 10% MMN sera diluted with DMEM containing 10% fetal bovine serum (FBS); MMN+TNF-α Ab: conditioned medium with 10% MMN sera pretreated with an anti-TNF-α neutralising antibody; MMN +IL-1β Ab: conditioned medium with 10% MMN sera pretreated with an anti-IL-1β neutralising antibody; MMN+IL-6 Ab: conditioned medium with 10% MMN sera pretreated with an anti-IL-6 neutralising antibody; MMN+TGF-β Ab: conditioned medium with 10% MMN sera pretreated with an anti-TGF-β neutralising antibody; MMN+GM6001: conditioned medium with 10% MMN sera pretreated with a GM6001; MMN+VEGF Ab: conditioned medium with 10% MMN sera pretreated with an anti-VEGF neutralising antibody. Control: non-conditioned DMEM containing 20% FBS; MMN: conditioned medium with 10% serum from a patient with MMN diluted with non-conditioned DMEM containing 10% FBS; Normal: conditioned medium with 10% serum from a healthy control diluted with non-conditioned medium of DMEM containing 10% FBS; GM1-IgM positive MMN: conditioned medium with 10% serum samples of MMN patients with anti-GM1 IgM antibodies; GM1-IgM negative MMN: conditioned medium with 10% serum samples of MMN patients without anti-GM1 IgM antibodies.

impaired remyelination caused by the disruption of the BNB was the mechanism responsible in this case. Oh *et al*[17] reported perivascular lymphocytic infiltration in the endoneurial or perineurial microvessels of the BNB in the motor nerves from an autopsy case of a patient with MMN. Thus, a leaky BNB that allows the intrusion of circulating pathogenic antibodies and inflammatory cytokines may play a crucial role in the development of MMN. Some studies have indeed demonstrated anti-GM1 antibody-mediated focal demyelination and blockade of voltage-dependent Na^+ channels at the node of Ranvier in vivo and in vitro[24–26] and reported that the sera obtained from patients with MMN can block nerve conduction in distal motor nerves in mice.[27] However, the molecular mechanism of BNB breakdown in MMN has not been adequately explained as yet. In the present study, we used conditionally immortalised human BNB-derived endothelial cells to analyse the effects of the sera from patients with MMN on the impairment of the BNB function.[14] We have also previously reported that VEGF disrupts the BNB and that sera obtained from patients with Bickerstaff's brainstem encephalitis and Miller Fisher syndrome did not influence the barrier



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function in the same in vitro BNB model.[21 28] Our present study is the first to demonstrate that the sera from patients with MMN can disrupt the BNB. The expression of claudin-5 and the TEER values were decreased, and the NaF permeability of PnMECs was increased after exposure to the MMN sera. Together, these results indicate that humoral factors in the MMN sera

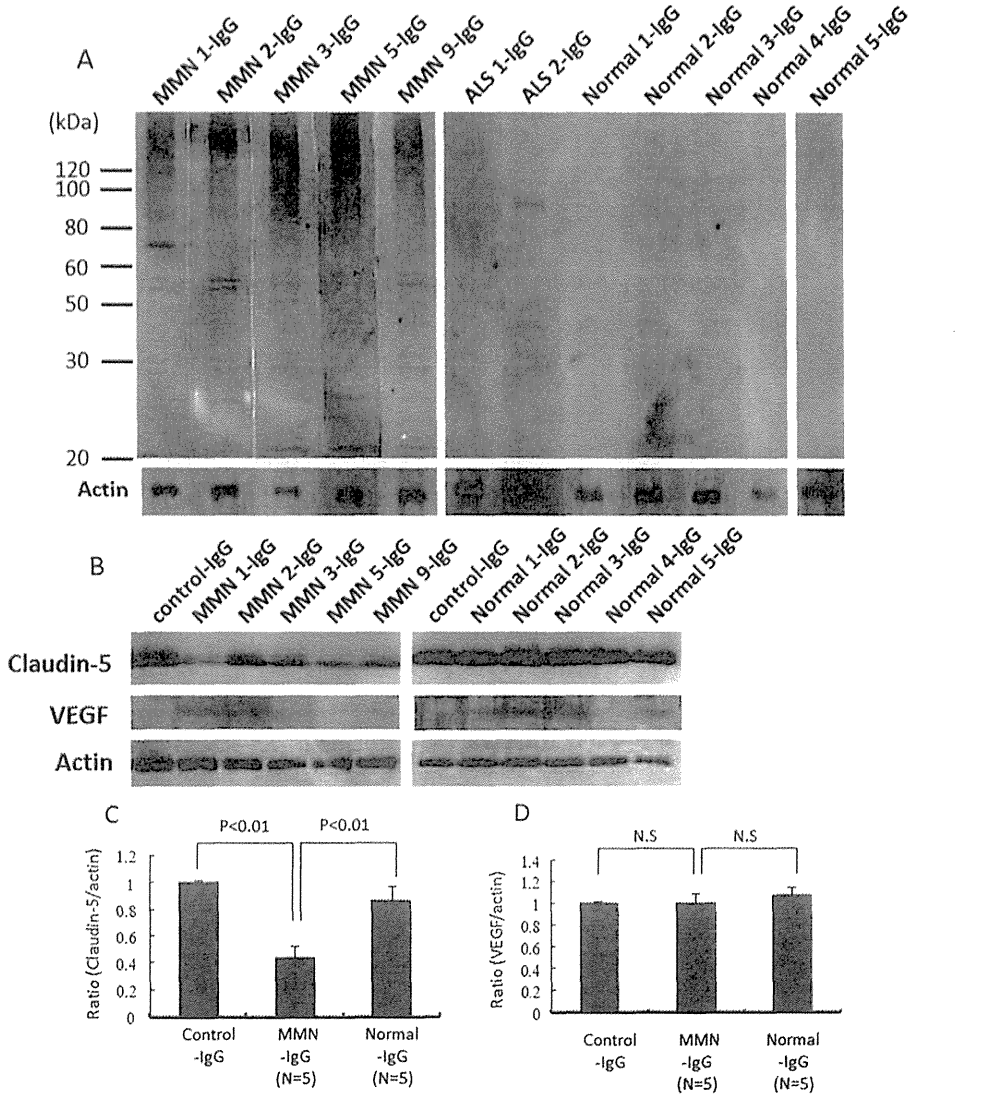
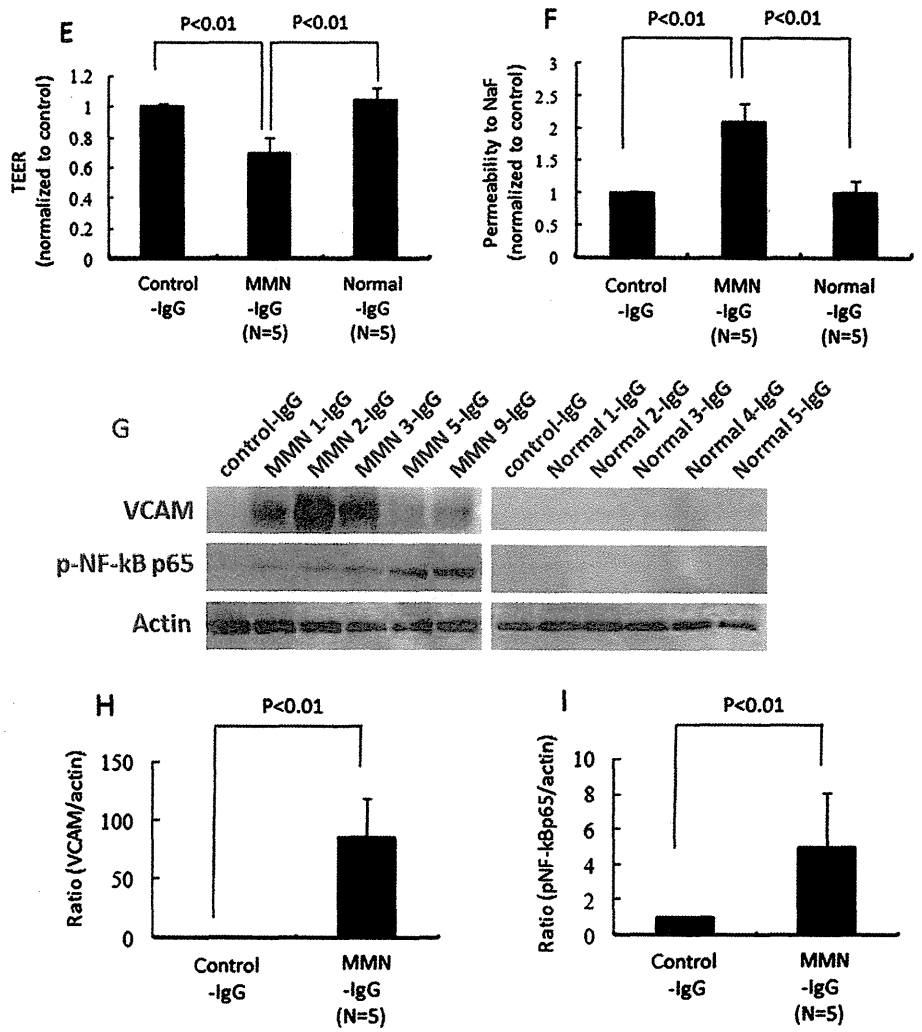


Figure 4 (A) Representative results obtained by immunoblotting of human peripheral nerve microvascular endothelial cell (PnMEC) lysates. The blots were exposed to the purified serum IgG from five patients with multifocal motor neuropathy (MMN), two patients with amyotrophic lateral sclerosis (ALS) and five healthy controls after loading 20 mg of protein lysates from PnMECs. The purified IgG fractions of the sera from patients with MMN predominantly reacted with one or more antigens of approximately 30, 45, 50, 54, 56 and 70 kDa in the PnMEC lysates. The purified serum IgG samples from the two patients with ALS also reacted with approximately 30, 40, 45, 50 and 90 kDa antigens of PnMECs. The two bands corresponding to the 40 and 45 kDa antigens of PnMECs were detected from the purified IgG fractions from the five healthy controls. The expression of actin was used as an internal standard. (B and C) The effects of the purified serum IgG from patients with MMN without anti-GM1 IgM antibodies on the expression of tight junction proteins and vascular endothelial growth factor (VEGF) in human peripheral nerve microvascular endothelial cells (PnMECs) were determined by a Western blot analysis. (B) The amount of claudin-5 in PnMECs was significantly decreased after exposure to the purified IgG fractions of patient's sera, whereas it was not affected by the purified IgG fractions from healthy controls, as determined by a Western blot analysis. The amount of VEGF proteins did not change following exposure to the purified serum IgG fractions obtained from the patients with MMN and healthy controls. (C and D) Each bar graph reflects the combined densitometry data from independent experiments (mean±SEM, n=5, *: $p<0.01$). The transendothelial electrical resistance value of PnMECs was significantly decreased (E) and the NaF permeability of PnMECs was significantly increased (F) after exposure to the purified IgG fraction from patients with MMN, although it was not changed by incubation with the purified IgG fractions from healthy controls. (G) The amount of VCAM-1 and NF-κB p65 in PnMECs was significantly increased after exposure to the purified IgG fraction from patients with MMN, whereas it was not changed by the purified IgG fractions from healthy controls, as determined by a Western blot analysis. (H and I) Each bar graph reflects the combined densitometry data from independent experiments (mean±SEM, n=5, $p<0.01$). Control-IgG: conditioned medium containing purified IgG fractions obtained from fetal bovine serum; MMN-IgG: conditioned medium with containing purified IgG fractions obtained from the sera of patients with MMN; ALS-IgG: conditioned medium with containing purified IgG fractions obtained from the sera of patients with ALS; Normal-IgG: conditioned medium with containing purified IgG fractions obtained from the sera of healthy individuals.

disrupt the BNB. We therefore first tried to identify the most important substance involved in disrupting the BNB in patients with MMN.

The presence of circulating cytokines, including TNF-α, IL-1β and VEGF, appears to be linked to the pathogenesis of the BNB breakdown in patients with MMN. Recent data suggest that



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these cytokines can disrupt the BNB; in particular, VEGF was able to induce BNB impairment.[21] Our present study demonstrated that the BNB function was restored after adding a neutralising anti-VEGF antibody to the MMN sera, indicating that VEGF was the key molecule responsible for the disruption of the BNB in the patients with MMN in our study. Although the serum concentration of VEGF was not increased in the patients with MMN compared to that from healthy controls, the secretion of VEGF by PnMECs was increased after exposure to the MMN sera. This finding suggests that the effect of VEGF occurred via an autocrine mechanism; thus, minimal secretion may lead to a significant effect. Our present studies demonstrated that the neutralising anti-VEGF antibody may also have therapeutic potential for restoring the BNB integrity in MMN. We were unable to identify which humoral factors in the MMN sera caused the increased VEGF secretion observed in the present study; however, we demonstrated that the amount of VEGF proteins did not change following exposure to IgG obtained from the MMN sera in our study, thus indicating that unknown humoral factors other than IgG in the MMN sera are key mediators of increased VEGF secretion (figure 5).

We next hypothesised that antibodies binding to PnMECs might be involved in the BNB disruption in patients with MMN, because antibody-mediated immunological therapies including high-dose IVIg are effective against MMN. We thus determined whether purified IgG from the MMN sera without complement would have a direct influence on the BNB properties. Our results demonstrated that the purified IgG from the MMN sera decreased the amount of claudin-5 and the TEER value, and increased the permeability of the BNB, thus indicating that unknown antibodies, possibly IgG, against PnMECs from the MMN sera cause the disruption of the BNB (figure 5). This finding supports our hypothesis concerning the etiopathogenesis of MMN: MMN has an antibody-mediated immunological basis.

The proportion of patients with MMN with anti-GM1 antibodies in our present study (~27%), was lower than that reported in several previous studies, in which these antibodies were detected in 22–85% of patients with MMN.[2] The wide variation observed in the incidence of these antibodies is likely derived from the different ELISA assays used in the different studies.[2–6] We believe that this fact did not influence the possible consequences of the interpretation of our present study because the absence of these antibodies does not exclude a diagnosis of MMN. The frequent presence of anti-GM1 IgM antibodies in the sera of patients with MMN and their decrease during improvement induced by cyclophosphamide[29–31] have

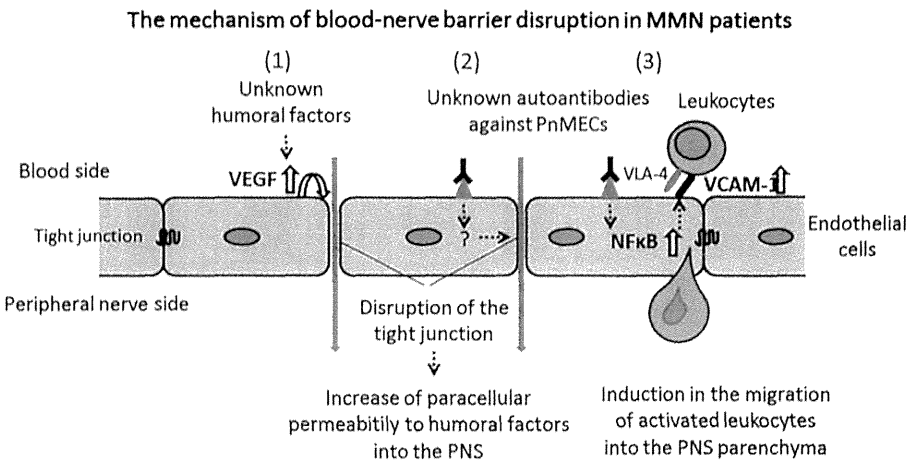

Figure 5 Schematic depiction of the assumed molecular mechanisms underlying the blood-nerve barrier (BNB) disruption observed in patients with multifocal motor neuropathy (MMN). The disruption of the BNB caused by humoral factors present in the sera of patients with MMN involves two differently regulated steps, including the disruption of tight junction proteins via the autocrine secretion of vascular endothelial growth factor from PnMECs induced by unknown humoral factors (1) and exposure to unknown autoantibodies against PnMECs (2), and the upregulation of VCAM-1 via NF-κB signalling in PnMECs induced by exposure to unknown autoantibodies against PnMECs in MMN sera (3).

favoured the hypothesis that GM1, which is present on the endothelial cells forming the BNB, may be the target of this immune response. GM1 is indeed present on the endothelial cells forming the BNB,[32] and anti-GM1 monoclonal antibodies can open the bovine BNB without the help of complement in vitro.[33] However, we found that the presence of anti-GM1 IgM antibodies in the patients' sera did not influence the BNB function in the PnMECs. Although the anti-GM1 IgM antibody is still a candidate cause of the disruption of the BNB, we consider that untested factors or unknown antibodies against PnMECs in the sera of patients with MMN other than the anti-GM1 IgM antibodies may be the key players that upset the BNB.

The interaction of VCAM-1 and very late activating antigen-4 (VLA-4) has a unique role in the pathogenesis of multiple sclerosis because it is involved in rolling and the arrest of leucocytes, which is a prerequisite for the activation of all further steps of transendothelial leucocyte migration.[34 35] We demonstrated that the sera from patients with MMN increased the amount of VCAM-1 protein, and this effect was reversed by exposure to an NF-κB inhibitor. The present study also has shown that the sera from patients with ALS had increased amounts of VCAM-1 protein. This finding can be explained by the hypothesis concerning a complicated pathogenesis of ALS, wherein immunological factors, including cytokines, chemokines and MMPs, and the disruption of the blood-brain barrier (BBB) and the blood-spinal cord barrier may play key roles in the development of the disease.[36 37] We also have indicated that the purified IgG of the sera from patients with MMN increased the amount of VCAM-1 proteins. This indicated that the unknown antibodies against PnMECs in the sera from patients with MMN may increase the VCAM-1 protein expression by upregulating the NF-κB signalling, thus causing the migration of activated leucocytes to the PNS parenchyma (figure 5), supporting the previous observation that perivascular lymphocytic infiltration in endoneurial microvessels of the BNB was present in autopsy cases of MMN.[17 18] Natalizumab is a humanised monoclonal antibody against the VLA-4 and inhibits the binding of leucocytes to the VCAM-1 expressed on activated brain vessels.[38 39] Our results provide the theoretical basis for applying natalizumab clinically in patients with MMN. In case of CIDP, natalizumab cannot be recommended at present, because Wolf *et al*[40] reported the case of a patient with CIDP in whom natalizumab treatment was not beneficial. Novel therapy directed specifically towards the reduction of VCAM-1 in the BNB could also be a possible therapeutic strategy for the treatment of MMN.

In conclusion, our study demonstrated that the disruption of the BNB caused by the humoral factors present in the sera of patients with MMN involves two differently regulated steps; the disruption of BNB function via the autocrine secretion of VEGF from PnMECs induced by unknown humoral factors or exposure to unknown autoantibodies against PnMECs, and the up-regulation of VCAM-1 in PnMECs induced by exposure to unknown autoantibodies against PnMECs in MMN sera. These data may provide novel explanations concerning the etiopathogenesis and the triggers of the BNB breakdown in MMN. A further analysis of the molecular mechanisms underlying the BNB breakdown observed in MMN, including the identification of the unknown molecules responsible for the disruption of BNB, and clarification of the effects of natalizumab and neutralising anti-VEGF antibodies against the disruption of the BNB, using an ex vivo or in vivo experimental model would assist in the development of therapies for this disabling disease.

Contributors FS performed the experiments, analysed and interpreted the data and wrote the manuscript. YS, TM and AT performed experiments and analysed the data. MO, NM and AM recruited patients. MK and RK evaluated the data and edited the manuscript. TK conducted and supervised the study, evaluated the data and wrote the manuscript.

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Competing interests All authors declare no potential conflicts of interest.

Patient consent Obtained.

Ethics approval The study was approved by the ethics committee of Yamaguchi University.

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REFERENCES

1 Parry GJ, Clarke S. Multifocal acquired demyelinating neuropathy masquerading as motor neuron disease. *Muscle Nerve* 1988;11:103–7.

2 Nobile-Orazio E. Multifocal motor neuropathy. *J Neuroimmunol* 2001;115:4–18.
3 Muley SA, Parry GJ. Multifocal motor neuropathy. *J Clin Neurosci* 2012;19:1201–9.
4 Meuth SG, Kleinschnitz C. Multifocal motor neuropathy: update on clinical characteristics, pathophysiological concepts and therapeutic options. *Eur Neurol* 2010;63:193–204.
5 Nobile-Orazio E, Cappellari A, Priori A. Multifocal motor neuropathy: current concepts and controversies. *Muscle Nerve* 2005;31:663–80.
6 Vlam L, van der Pol WL, Cats EA, *et al*. Multifocal motor neuropathy: diagnosis, pathogenesis and treatment strategies. *Nat Rev Neurol* 2011;8:48–58.
7 van der Pol WL, Cats EA, van den Berg LH. Intravenous immunoglobulin treatment in multifocal motor neuropathy. *J Clin Immunol* 2010;30:S79–83.
8 Umapathi T, Hughes RA, Nobile-Orazio E, *et al*. Immunosuppressant and immunomodulatory treatments for multifocal motor neuropathy. *Cochrane Database Syst Rev* 2012;(4):CD003217.
9 Van Asseldonk JT, Franssen H, Van den Berg-Vos RM, *et al*. Multifocal motor neuropathy. *Lancet Neurol* 2005;4:309–19.
10 Yuki N, Watanabe H, Nakajima T, *et al*. IVIG blocks complement deposition mediated by anti-GM1 antibodies in multifocal motor neuropathy. *J Neurol Neurosurg Psychiatry* 2011;82:87–91.
11 Susuki K, Rasband MN, Tohyama K, *et al*. Anti-GM1 antibodies cause complement-mediated disruption of sodium channel clusters in peripheral motor nerve fibers. *J Neurosci* 2007;27:3956–67.
12 Willison HJ, Halstead SK, Beveridge E, *et al*. The role of complement and complement regulators in mediating motor nerve terminal injury in murine models of Guillain-Barré syndrome. *J Neuroimmunol* 2008;15:172–82.
13 Poduslo JF, Curran GL, Berg CT. Macromolecular permeability across the blood-nerve and blood-brain barriers. *Proc Natl Acad Sci USA* 1994;91:5705–9.
14 Abe M, Sano Y, Maeda T, *et al*. Establishment and characterization of human peripheral nerve microvascular endothelial cell lines: a new in vitro blood-nerve barrier (BNB) model. *Cell Struct Funct* 2012;37:89–100.
15 Kanda T, Numata Y, Mizusawa H. Chronic inflammatory demyelinating polyneuropathy: decreased claudin-5 and relocated ZO-1. *J Neurol Neurosurg Psychiatry* 2004;75:765–9.
16 Kaji R, Hirota N, Oka N, *et al*. Anti-GM1 antibodies and impaired blood-nerve barrier may interfere with remyelination in multifocal motor neuropathy. *Muscle Nerve* 1994;17:108–10.
17 Oh SJ, Claussen GC, Odabasi Z, *et al*. Multifocal demyelinating motor neuropathy: pathologic evidence of 'inflammatory demyelinating polyradiculoneuropathy'. *Neurology* 1995;45:1828–32.
18 Taylor BV, Dyck PJ, Engelstad J, *et al*. Multifocal motor neuropathy: pathologic alterations at the site of conduction block. *J Neuropathol Exp Neurol* 2004;63:129–37.
19 van Schaik IN, Léger JM, Nobile-Orazio E, *et al*. Joint Task Force of the EFNS and the PNS.Joint Task Force of the EFNS and the PNS. European Federation of Neurological Societies/Peripheral Nerve Society guideline on management of multifocal motor neuropathy. Report of a joint task force of the European Federation of Neurological Societies and the Peripheral Nerve Society—first revision. *J Peripher Nerv Syst* 2010;15:295–301.
20 Brooks BR, Miller RG, Swash M, *et al*.; World Federation of Neurology Research Group on Motor Neuron Diseases. El Escorial revisited: revised criteria for the diagnosis of amyotrophic lateral sclerosis. *Amyotroph Lateral Scler Other Motor Neuron Disord* 2000;1:293–9.
21 Shimizu F, Sano Y, Abe MA, *et al*. Peripheral nerve pericytes modify the blood-nerve barrier function and tight junctional molecules through the secretion of various soluble factors. *J Cell Physiol* 2011;226:255–66.
22 Corse AM, Chaudhry V, Crawford TO, *et al*. Sensory nerve pathology in multifocal motor neuropathy. *Ann Neurol* 1996;39:319–25.
23 Bouche P, Moulonguet A, Younes-Chennoufi AB, *et al*. Multifocal motor neuropathy with conduction block: a study of 24 patients. *J Neurol Neurosurg Psychiatry* 1995;59:38–44.
24 Santoro M, Uncini A, Corbo M, *et al*. Experimental conduction block induced by serum from a patient with anti-GM1 antibodies. *Ann Neurol* 1992;31:385–90.
25 Arasaki K, Kusunoki S, Kudo N, *et al*. Acute conduction block in vitro following exposure to antiganglioside sera. *Muscle Nerve* 1993;16:587–93.
26 Takigawa T, Yasuda H, Kikkawa R, *et al*. Antibodies against GM1 ganglioside affect K+ and Na+ currents in isolated rat myelinated nerve fibers. *Ann Neurol* 1995;37:436–42.
27 Roberts M, Willison HJ, Vincent A, *et al*. Multifocal motor neuropathy human sera block distal motor nerve conduction in mice. *Ann Neurol* 1995;38:111–18.
28 Saito K, Shimizu F, Koga M, *et al*. Blood-brain barrier destruction determines Fisher/Bickerstaff clinical phenotypes: an in vitro study. *J Neurol Neurosurg Psychiatry* 2013;84:756–65.
29 Brannagan TH III, Alaedini A, Gladstone DE. High-dose cyclophosphamide without stem cell rescue for refractory multifocal motor neuropathy. *Muscle Nerve* 2006;34:246–50.
30 Pestronk A, Cornblath DR, Ilyas AA, *et al*. A treatable multifocal motor neuropathy with antibodies to GM1 ganglioside. *Ann Neurol* 1988;24:73–8.
31 Chaudhry V, Corse AM, Cornblath DR, *et al*. Multifocal motor neuropathy: response to human immune globulin. *Ann Neurol* 1993;33:237–42.
32 Kanda T, Iwasaki T, Yamawaki M, *et al*. Isolation and culture of bovine endothelial cells of endoneurial origin. *J Neurosci Res* 1997;49:769–77.
33 Kanda T, Iwasaki T, Yamawaki M, *et al*. Anti-GM1 antibody facilitates leakage in an in vitro blood-nerve barrier model. *Neurology* 2000;55:585–7.
34 Engelhardt B. T cell migration into the central nervous system during health and disease: different molecular keys allow access to different central nervous system compartments. *Clin Exp Neuroimmunol* 2010;1:79–93.
35 Takeshita Y, Ransohoff RM. Inflammatory cell trafficking across the blood-brain barrier: chemokine regulation and in vitro models. *Immunol Rev* 2012;248:228–39.
36 Tarasiuk J, Kułakowska A, Drozdowski W, *et al*. CSF markers in amyotrophic lateral sclerosis. *J Neural Transm* 2012;119:747–57.
37 Garbuzova-Davis S, Rodrigues MC, Hernandez-Ontiveros DG, *et al*. Amyotrophic lateral sclerosis: a neurovascular disease. *Brain Res* 2011;1398:113–25.
38 Engelhardt B, Kappos L. Natalizumab: targeting alpha4-integrins in multiple sclerosis. *Neurodegener Dis* 2008;5:16–22.
39 Miller DH, Soon D, Fernando KT, *et al*., AFFIRM Investigators. MRI outcomes in a placebo-controlled trial of natalizumab in relapsing MS. *Neurology* 2007;68:1390–401.
40 Wolf C, Menge T, Stenner MP, *et al*. Natalizumab treatment in a patient with chronic inflammatory demyelinating polyneuropathy. *Arch Neurol* 2010;67:881–3.

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Severity and Patterns of Blood-Nerve Barrier Breakdown in Patients with Chronic Inflammatory Demyelinating Polyradiculoneuropathy: Correlations with Clinical Subtypes

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Abstract

Objective: Chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) is currently classified into clinical subtypes, including typical and atypical forms (multifocal acquired demyelinating sensory and motor neuropathy (MADSAM) and distal acquired demyelinating symmetric neuropathy (DADS)). The aim of this study was to elucidate the patterns and severity of breakdown of the blood-nerve barrier (BNB) in each CIDP subtype.

Methods: We evaluated the effects of sera obtained from patients with typical CIDP, MADSAM and DADS and control subjects on the expression levels of tight junction proteins and transendothelial electrical resistance (TEER) value in human peripheral nerve microvascular endothelial cells (PnMECs).

Results: The sera obtained from the patients with the three clinical phenotypes of CIDP decreased the amount of claudin-5 protein levels and TEER values in the PnMECs. In addition, the sera obtained from typical CIDP patients more prominently reduced claudin-5 protein levels and TEER values in the PnMECs than did that obtained from the MADSAM and DADS patients. Furthermore, the severity of BNB disruption after exposure to the sera was associated with higher Hughes grade, lower MRC score, more pronounced slowing of motor nerve conduction in the median nerve and higher frequency of abnormal temporal dispersion.

Conclusions: Sera derived from typical CIDP patients destroy the BNB more severely than those from MADSAM or DADS patients. The extent of BNB disruption in the setting of CIDP is associated with clinical disability and demyelination in the nerve trunk. These observations may explain the phenotypical differences between CIDP subtypes.

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Introduction

Chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) is a rare autoimmune-mediated neuropathy thought to constitute a group of heterogeneous disorders involving a wide range of clinical phenotypes, variable clinical course and differing responses to immunotherapy [1,2]. The Joint Task Force of the European Federation of Neurological Societies and Peripheral Nerve Society (EFNS/PNS) convened in 2010 divided CIDP into two clinical subtypes: "typical CIDP (t-CIDP)," the classical pattern of CIDP, and "atypical CIDP," which include multifocal acquired demyelinating sensory and motor neuropathy (MADSAM) and distal acquired demyelinating symmetric neuropathy (DADS) [3]. t-CIDP is clinically defined by the presence of chronically progressive or recurrent symmetrical proximal and distal weakness and sensory dysfunction in all extremities developing over at least two months and likely affects a relatively uniform group of patients [4,5]. In contrast, MADSAM neuropathy is characterized by an asymmetrical multifocal pattern of motor and sensory impairment (mononeuropathy multiplex) likely representing an asymmetrical variant of CIDP [6,7]. On the other hand, DADS neuropathy is characterized by symmetrical sensory

and motor polyneuropathy of the distal upper and lower limbs predominantly associated with muscle weakness and/or sensory disturbances in the distal limbs [8,9]. These three CIDP subtypes share a common feature, namely, chronic demyelinative neuropathy of supposed immune origin; however, the different clinical phenotypes appear to result from differences in the underlying immunopathogenesis [10].

Various previous reports have demonstrated that the pathological breakdown of the blood-nerve barrier (BNB), which allows for the entry of immunoglobulins, cytokines and chemokines into the peripheral nerve system (PNS) parenchyma, is a key event in the disease process of CIDP [11,12,13], and the result of electrophysiological examinations have led to a new hypothesis concerning the pathogenesis of CIDP, namely that differences in the degree of BNB malfunction partly determine the differences in both the distribution of demyelinative lesions and clinical phenotypes observed between t-CIDP and MADSAM neuropathy [10,14,15]. In the present study, we evaluated the contributions of humoral factors in sera obtained from patients with each clinical subtype of CIDP to BNB breakdown and clarified the association between BNB disruption and clinical profiles using our previously established human BNB-derived immortalized endothelial cells [16].

Materials and methods

Serum and cerebrospinal fluid samples

The study protocol was approved by the ethics committee of Yamaguchi University and Chiba University. All patients consented to participate and written informed consent was obtained from each subject. Serum was collected from a total of 25 CIDP patients with t-CIDP (n = 12), MADSAM (n = 10) and DADS (n = 3) in the initial progressive phase of the disease or at relapse, without either corticosteroid or intravenous immunoglobulin (IVIg) treatment, diagnosed at Chiba University Hospital or Yamaguchi University Hospital. All patients fulfilled the diagnostic criteria for CIDP based on the guidelines reported by the EFNS/PNS 2010 [3]. The inclusion criteria was a diagnosis of definitive or probable CIDP. None of the patients with DADS had anti-myelin-associated glycoprotein (MAG) antibodies. Sera obtained from 10 healthy individuals served as normal controls. All serum samples were inactivated at 56°C for 30 minutes just prior to use. Cerebrospinal fluid (CSF) samples obtained from the 25 patients with CIDP were analyzed with respect to the protein level in the CSF, the IgG index and/or CSF/serum albumin ratio (Q Alb). The clinical and electrophysiological data for all CIDP patients were analyzed. The clinical parameters included the Hughes functional grading scale [17], which was used as a functional assessment, and the total Medical Research Council (MRC) scale for four muscle groups (deltoid, wrist extensor, iliopsoas and tibialis anterior muscles). All 25 patients received immune system-modulating treatment, including corticosteroids and IVIg. Treatment was considered to be effective if the patient's condition, including the Hughes scale and MRC score, was found to have improved after therapy. Nerve conduction studies were performed according to conventional procedures and using standard electromyography machine (Neuropack M1, Nihon Kohden, Tokyo, Japan; Viking 4, Nicolet Biomedical Japan, Tokyo, Japan). Motor nerve studies of the median, ulnar and tibial nerves were performed, including F wave analyses. The terminal latency index (TLI) was calculated based on the following formula: TLI = terminal distance (mm)/(distal latency (ms) × conduction velocity (m/s)). A partial motor conduction block was defined as a more than a 50% reduction in the compound muscle action potentials (CMAP) between the stimulus sites, and abnormal temporal dispersion was defined as a more than 30% increase in duration between the proximal and distal CMAP, in accordance with the EFNS/PNS guidelines [3].

Cell culture and treatment

Immortalized human peripheral nerve microvascular endothelial cells (PnMECs), termed "FH-BNBs", were generated previously in our laboratory [16]. The cells were cultured in medium [Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS; Sigma, St. Louis, MO, U.S.A) and antibiotics] with 10% patient serum or culture medium containing 10% FBS, which was used as a control, in an incubator at 37°C with 5% CO_2/air. The cells were maintained for either 24 hours to measure the transendothelial electrical resistance (TEER) value or 48 hours to extract total proteins.

Reagents

We purchased polyclonal anti-claudin-5 and anti-occludin antibodies from Zymed (San Francisco, CA, U.S.A). Polyclonal anti-actin antibodies were purchased from Santa Cruz (Santa Cruz, CA, U.S.A).

Western blot analysis

After boiling, aliquots containing equal amounts of protein (15 μg) were separated via SDS-PAGE (Bio-Rad, Hercules, CA). The proteins were then transferred onto nitrocellulose membranes (Amersham, Chalfont, UK), as previously described [18]. The membranes were subsequently treated with the relevant primary antibodies (dilution: 1:100) for two hours and then incubated with the secondary antibodies (dilution: 1:2,000) for one hour at room temperature. Finally, the proteins were visualized using an enhanced chemiluminescence detection system (ECL-prime, Amersham, UK). The optical density of each band was assessed using the Quantity One software program (Bio-Rad).

Transendothelial electrical resistance (TEER) studies

The TEER values in the cell layers were measured using a Millicell electrical resistance apparatus (Endohm-6 and EVOM, World Precision Instruments, Sarasota, FL, U.S.A), according to the manufacturer's instructions. The cells were seeded (1×10^6 cells/insert) on collagen-coated Transwell inserts (pore size: 0.4 μm, effective growth area: 0.3 cm^2, BD Bioscience, Sparks, MD, USA), and the TEER value for each insert was calculated following treatment with each type of medium (non-conditioned medium was used as a control, the conditioned medium contained 10% patient serum) for 24 hours by subtracting the blank from each reading. Each condition was tested in triplicate for each experiment.

Data analysis

Differences in the median values between the groups were examined according to the Mann-Whitney U test, with two-sided P value of <0.05 considered to be statistically significant. Pearson correlation coefficients were used to test the associations. All statistical analyses were performed using the IBM SPSS statistical software program, version 21J.

Results

Clinical characteristics

The clinical profiles of patients with t-CIDP, MADSAM and DADS are summarized in Table 1. The mean Hughes grade was

significantly higher in the t-CIDP patients than in the MADSAM or DADS patients and in the MADSAM patients than in the DADS patients. In addition, significantly lower mean MRC values for both the total score for the four muscle groups and the iliopsoas alone were observed in the t-CIDP patients compared to those noted in the MADSAM and DADS patients. Meanwhile, the mean CSF protein concentration was higher in the t-CIDP and DADS patients than in the MADSAM patients. Based on the results of the electrophysiological examinations of the median nerve, the t-CIDP and DADS patients demonstrated a more prolonged average motor nerve distal latency than the MADSAM patients, and while the t-CIDP patients displayed greater slowing of mean motor nerve conduction than the MADSAM patients. Furthermore, a higher frequency of conduction block was observed in the MADSAM patients than in the t-CIDP patients. In contrast, the MADSAM patients exhibited temporal dispersion much less frequently than did the t-CIDP and DADS patients.

The sera obtained from the patients with t-CIDP, MADSAM and DADS disrupted the BNB

We first examined the effects of the sera obtained from the patients with the three clinical subtypes of CIDP on the expression levels of tight junction proteins and the TEER values in the FH-BNBs. Consequently, the protein ratio of claudin-5 to actin proteins was significantly lower in the FH-BNBs exposed to sera from the patients with t-CIDP, MADSAM and DADS than in those incubated with sera from the healthy controls, as determined in a Western blot analysis (**Figs. 1 A–E**). In contrast, the ratio of occludin to actin proteins in the FH-BNBs did not change after a challenge with the sera obtained from the CIDP patients or healthy controls (**Figs. 1A–D, F**). Meanwhile, the TEER values in the FH-BNBs were significantly decreased following exposure to the sera obtained from the t-CIDP, MADSAM and DADS patients in comparison to that observed after exposure to sera of the healthy control (**Fig. 1G**). Furthermore, the ratio of claudin-5 to actin proteins and the TEER values observed after exposure to the sera obtained from t-CIDP patients were significantly lower than those observed after exposure to the sera obtained from the MADSAM and DADS patients (**Figs. 1E, G**). Moreover, the TEER values observed after exposure to the sera obtained from the DADS patients were significantly lower than those observed after exposure to the sera obtained from the MADSAM patients, although the ratio of claudin-5 to actin proteins was not significantly different between the two groups (**Figs. 1E, G**).

Correlations between the clinical, laboratory and electrophysiological findings and BNB malfunction in the patients with CIDP

We next examined the associations between the clinical, laboratory and electrophysiological findings and the ratio of claudin-5 to actin proteins and/or the TEER values in the FH-BNBs exposed to the sera from the CIDP patients. Consequently, the decrease in either the claudin-5 protein level or TEER value in the FH-BNBs was found to be associated with the clinical severity.

Table 1.

	t-CIDP (n = 12)	MADSAM (n = 10)	DADS (n = 3)	
	Mean (±SD), Percent [number]	Mean (±SD), Percent [number]	Mean (±SD), Percent [number]	p Value
Clinical profile				
Age (year)	56 (±12)	56 (±12)	53 (±6)	NS
Male: Female	9:3	8:2	2:1	NS
Disease duration (year)	5.2 (±6.9)	5.4 (±5.4)	3.2 (±1)	NS
Hughes grade scale	2.83 (±0.94)	1.7 (±1.06)	1 (±0)	0.011*, 0.007**, 0.037***
Response to treatment	67% [8/12]	80% [8/10]	100% [3/3]	NS
MRC score				
Total (deltoid+ wrist extensor + iliopsoas + tibialis anterior)	15.7 (±2.6)	18.3 (±1.5)	19.7 (±0.6)	0.015*, 0.013**
CSF protein (mg/dl)	95.3 (±34.7)	55.8 (±31.4)	114.9 (±63.7)	0.002*, 0.028***
CSF IgG index	0.610 (±0.099)	0.560 (±0.134)	0.573 (±0.016)	NS
CSF Q Albumin	0.028 (±0.047)	0.009 (±0.006)	0.014 (±0.011)	NS
Motor conduction study				
Median nerve				
Distal latency (ms)	9.0 (±5.9)	4.7 (±1.0)	14.3 (±12.7)	0.016*, 0.012***
Conduction velocity (m/s)	30.2 (±12.6)	42.3 (±8.3)	41.3 (±0.3)	0.033*
CMAP (mV)	4.6 (±3.4)	6.1 (±3.5)	5.3 (±3.5)	NS
Terminal latency index	0.34 (±0.13)	0.39 (±0.18)	0.18 (±0.11)	NS
Conduction block	58% [7/12]	100% [10/10]	67% [2/3]	0.020*
Temporal dispersion	80% [8/10]	30% [3/10]	100% [3/3]	0.018*, 0.017***

*t-CIDP vs MADSAM, **t-CIDP vs DADS, ***MADSAM vs DADS.
Data are expressed as mean (±SD), median [range] or percent {number}.
t-CIDP, typical chronic inflammatory demyelinating polyradiculoneuropathy; MADSAM, multifocal acquired demyelinating sensory and motor neuropathy; DADS, distal acquired demyelinating symmetric neuropathy, IVIg: Intravenous immunoglobulin, MRC: Medical Research Council, CSF: cerebrospinal fluid, CMAP: compound muscle action potential.
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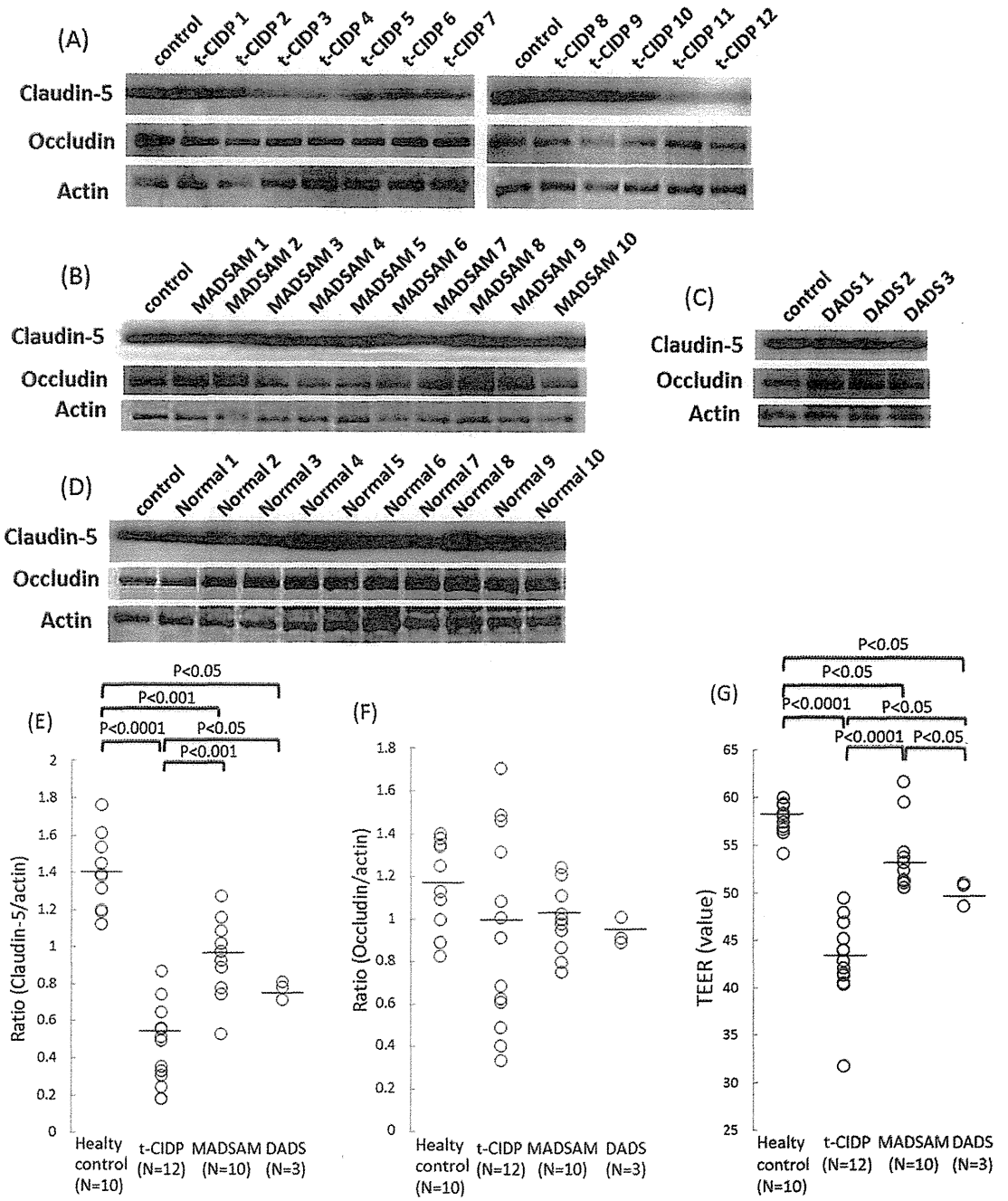

Figure 1. The sera obtained from the patients with t-CIDP, MADSAM and DADS disrupted the BNB. (A) – (D) Effects of the sera obtained from patients with three different phenotypes of chronic inflammatory demyelinating polyneuropathy (CIDP) on the protein levels of claudin-5 and occludin in the FH-BNBs, as determined using a Western blot analysis. The cells were exposed to sera from either patients with typical CIDP (t-CIDP) (A), multifocal acquired demyelinating sensory and motor neuropathy (MADSAM) (B) or distal acquired demyelinating symmetric neuropathy (DADS) (C) or healthy volunteers (D). (E) The sera obtained from the patients with t-CIDP, MADSAM neuropathy and DADS neuropathy decreased the protein ratio of claudin-5 to actin proteins in the FH-BNBs compared to that observed following exposure to the sera from the healthy volunteers. The decrease in the claudin-5 levels in the FH-BNBs was greater after incubation with the sera obtained from the t-CIDP patients than after that with the sera from the patients with MADSAM and DADS. (F) There were no significant differences between the patients with the three different phenotypes of CIDP and the healthy controls regarding the occludin protein levels in the FH-BNBs. (G) The effects of the sera on the transendothelial electrical resistance (TEER) values in the FH-BNBs were also evaluated. Adding sera obtained from the patients with t-CIDP, MADSAM neuropathy or DADS neuropathy resulted in decreased TEER values in the FH-BNBs in comparison with that observed in the cells treated with the sera obtained from the healthy volunteers. Markedly decreased TEER values in FH-BNBs were also observed in the FH-BNBs following incubation with the sera obtained from the t-CIDP patients compared to that noted in the cells incubated with sera from patients with MADSAM or DADS neuropathy. The TEER values were decreased following exposure to the sera obtained from the patients with DADS neuropathy compared to that observed after exposure to the sera obtained from the patients with MADSAM neuropathy. The bars indicate the mean level in each group. Control: non-conditioned DMEM containing 20% FBS. t-CIDP: conditioned medium with 10% sera obtained from patients with t-CIDP diluted with non-conditioned DMEM containing 10% FBS. MADSAM: conditioned medium with 10% sera obtained from patients with MADSAM diluted with non-conditioned DMEM containing 10% FBS. DADS: conditioned medium with 10% sera obtained from patients with DADS diluted with non-conditioned DMEM containing 10% FBS. Normal: conditioned medium with 10% sera obtained from a healthy volunteer diluted with non-conditioned medium of DMEM containing 10% FBS.
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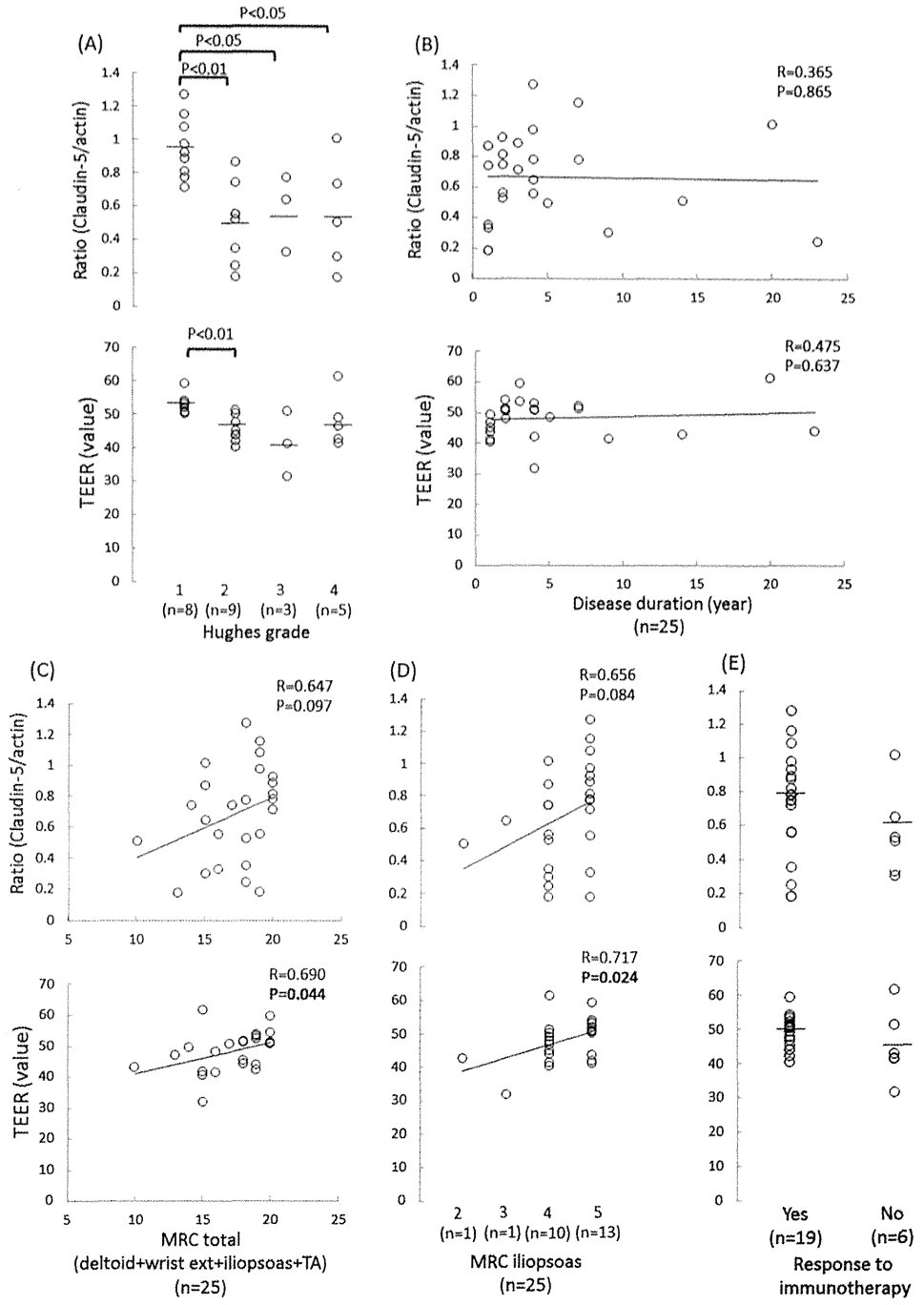

Figure 2. Associations between the clinical findings and BNB malfunction in the patients with CIDP. Correlations between the claudin-5 to actin protein ratios and the TEER values in the FH-BNBs following exposure to sera and the clinical parameters in the patients with CIDP. Associations between the claudin-5 to actin protein ratios and TEER values and the Hughes grade (A), duration of disease from onset (B), total Medical Research Council (MRC) scores for four muscle groups (deltoid, wrist extensor, iliopsoas, and tibialis anterior muscles) (C), MRC score for the iliopsoas muscle (D) and response to treatment, including intravenous immunoglobulin (IVIg) and corticosteroids (E). A lower ratio of claudin-5 to actin proteins was significantly associated with a higher Hughes grade, while a lower TEER value significantly correlated with a higher Hughes grade and lower MRC score.

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In addition, a lower ratio of claudin-5 to actin proteins significantly correlated with a higher Hughes grade (**Fig. 2A**) and higher Q Alb level (**Fig. 3C**), while a lower TEER value was significantly associated with a higher Hughes grade (**Fig. 2A**), lower MRC score (**Fig. 2C**), particularly in the iliopsoas muscle (**Fig. 2D**), more pronounced slowing of the motor nerve conduction in the median nerve (**Fig. 4B**) and higher frequency of abnormal temporal dispersion (**Fig. 4F**). In contrast, no significant differences were noted between the claudin-5 to actin protein ratio or TEER value and the duration of disease from onset (**Fig. 2B**), response to immunotherapy (**Fig. 2E**), concentration of CSF proteins (**Fig. 3A**), IgG index (**Fig. 3B**), distal latency (**Fig. 4A**),

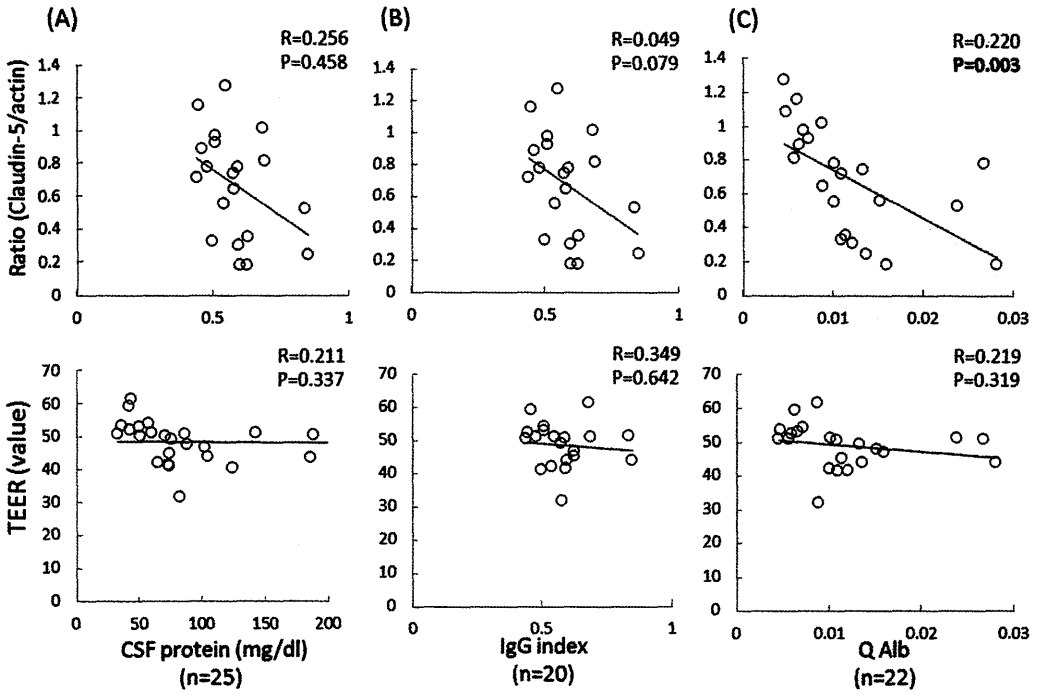

Figure 3. Associations between the CSF parameters and BNB disruption in the patients with CIDP. Correlations between the claudin-5 to actin protein ratios and the TEER values in the FH-BNBs following exposure to sera and the cerebrospinal fluid (CSF) parameters, including the CSF protein level (A), IgG index (B) and albumin ratio (Q Alb) (C) in the patients with CIDP. A lower ratio of claudin-5 to actin proteins was significantly associated with a higher Q Alb.
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conduction block (Fig. 4E) or CMAP amplitude (**Fig. 4C**) or TLI index (**Fig. 4D**) in the median nerve.

Discussion

According to the 2010 EFNS/PNS guidelines, CIDP comprises several clinical subtypes, including t-CIDP, MADSAM and DADS, based on the distribution of signs and symptoms [3]. Electrophysiological examinations provide important information regarding the pathogenesis of CIDP, as the distribution patterns of demyelinating lesions differ substantially between the different clinical phenotypes of CIDP [14]. These observations prompted us to hypothesize that differences in the patterns of BNB disruption at least partly determine the distribution of demyelinating lesions and clinical phenotypes of CIDP [10]. In cases of t-CIDP, motor nerve conduction studies frequently show a prolonged distal latency or duration of the distal CMAP, suggesting that demyelination predominantly may affect the distal nerve terminals, where the BNB is most vulnerable, during the initial phase of the disease [10,14]. However, demyelination also affects the intermediate nerve trunk after a long course of disease in individuals with t-CIDP, due to gradual disruption of the BNB in the nerve trunk. This phenomenon reflects profound slowing of nerve conduction, conduction block and/or abnormal temporal dispersion in the intermediate nerve segments, as identified on motor nerve conduction studies [10]. These disease processes suggest the importance of BNB breakdown in the development of t-CIDP. In contrast, electrophysiology studies of MADSAM have characterized the disease as involving multifocal nerve conduction block in the intermediate nerve trunks, with preservation of the nerve terminals and roots [10,19], suggesting the presence of multifocal demyelination in these regions. The pattern of BNB disruption appears to differ between MADSAM and t-CIDP, as the multifocal breakdown of the BNB at the site of conduction block may be required for the development of the former condition [10]. The hypothesis suggested by the findings of an electrophysiological studies is of great interest because it may explain the clinical variety of CIDP; however, it is not adequately supported by the results of pathological or cell biological examinations. Only one report regarding pathological changes in the endoneurial microvessels of patients with CIDP has been published to date [11]. This report described the characteristic of pathological changes in tight junction proteins, including a decrease in the level of claudin-5 and altered localization of ZO-1 on sural nerve biopsy samples obtained from t-CIDP patients. However, it remains unclear whether breakdown of the BNB is involved in the pathogenesis of atypical CIDP.

In the present study, we used our previous established human BNB-derived endothelial cells [16] and assessed the degree of BNB damage following exposure to sera by calculating the changes in the protein ratio of claudin-5 to actin proteins and measuring the TEER value [18]. Our results demonstrated that the sera obtained from the patients with three clinical phenotypes of CIDP all significantly decreased claudin-5 expression and the TEER value in the FH-BNBs, suggesting that humoral factors present in the sera of MADSAM and DADS patients, as well as t-CIDP patients, induce the BNB malfunction. The decrease in the claudin-5 protein level and TEER values observed following exposure to the sera obtained from the t-CIDP patients was more remarkable than that observed after incubation with the sera obtained from the patients with MADSAM or DADS. These findings indicate that the severity of BNB breakdown differs depending on the clinical phenotype of CIDP; humoral factors in the sera of t-CIDP patients may cause more severe BNB damage than those present in the