Table 2
Treatments and INCAT scores of patients with anti-MAG neuropathy.

Patients	Treatment					Follow-up period (months)	Pre-INCAT	Post-INCAT	ΔINCAT	INCAT progression index
	Rituximab	IVIg	Plasma exchange	Prednisolone	Other					
1		+				28	4	4	0	0.00
2		+				12	3	6	3	0.25
3		+	+			62	4	6	2	0.03
4	+		+			18	3	3	0	0.00
5		+				60	3	2	−1	−0.02
6	+	+		+		12	4	4	0	0.00
7		+		+		60	3	7	4	0.07
8				+	Melphalan	30	3	7	4	0.13
9		+	+	+		17	2	3	1	0.06
10		+				29	1	1	0	0.00
11		+			Cyclophosphamide	37	3	2	−1	−0.03
12		+		+		15	5	3	−2	−0.13
13		+		+	Tacrolimus	5	5	5	0	0.00
14		+		+		44	2	2	0	0.00
15	+	+		+		34	3	2	−1	−0.03
16		+		+		45	4	1	−3	−0.07
17	+	+		+		16	1	1	0	0.00
18		+				7	5	8	3	0.43
19		+				41	4	1	−3	−0.07

Pre-INCAT, the INCAT score before treatment; post-INCAT, the INCAT score after treatment.

anti-MAG neuropathy were also found to bind to MAG and phosphacan with varied reactivities.

We then examined the clinical relevance of the observed distinct antibody reactivities, and found that the P/M ratio (the ratio of the antibody activities against phosphacan to MAG) was significantly correlated with ΔINCAT score and with the INCAT score progression index, indicating that strong reactivities of anti-MAG antibodies to phosphacan were associated with treatment resistance or progressive clinical courses. Consistently, the ΔINCAT score and the INCAT score progression index associated with a P/M ratio ≥1.0 were significantly higher than those with a P/M ratio <1.0. The IgMs in the patients should recognize the HNK-1 epitope because they showed no reactivity to MAG or phosphacan without the HNK-1 epitope. We cannot conclude that treatment-responsiveness or prognosis is associated with the specificity of anti-HNK-1 antibodies, since various treatment regimens were used in this retrospective study. Nonetheless, outcomes did not seem associated with any specific treatment; even only IVIg or IVIg plus prednisolone was associated with good response, but it was also associated with poor response. Rituximab has been shown to alleviate the symptoms of patients with anti-MAG neuropathy in uncontrolled series (Motoyama et al., 2011; Pestronk et al., 2003; Shimoyama et al., 2011), but negative results for primary endpoints were obtained in two recent double-blind, randomized, placebo-controlled trials of rituximab for anti-MAG neuropathy (Dalakas et al., 2009; Leger et al., 2013). We cannot also conclude the effectiveness of rituximab, since only one of four patients responded to this medicine. From these findings together with the current results, we speculate that the treatment-responsiveness or prognosis of patients is not associated with the specific treatment, but rather that it is associated with the observed antibody specificities.

This study did not prove that phosphacan is implicated directly to pathological processes in neuropathy. Nevertheless, this protein is important for central and peripheral nervous systems, since it is a major chondroitin sulfate proteoglycan that is involved in the modulation of cell adhesion and neurite outgrowth during neural development and regeneration (Faissner et al., 2006). A recent study showed that phosphacan is involved in the blockade of CNS regeneration (Buss et al., 2009), but this blocking effect depends on the neuronal lineage, suggesting that phosphacan allows only appropriate neurons to regenerate (Garwood et al., 1999). An *in vitro* study of adult rat dorsal root ganglion (DRG) neurons

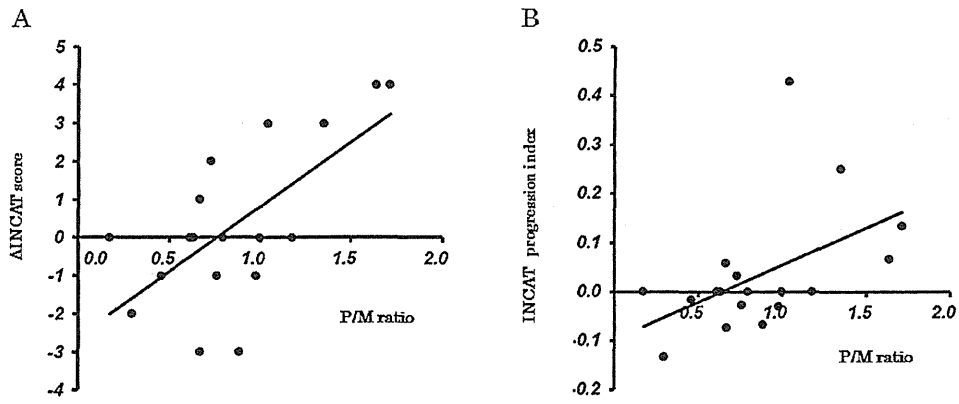

Fig. 2. (A) The ΔINCAT score (the increase in the INCAT score) had a positive correlation with the ratio of anti-phosphacan to anti-MAG antibody activities (P/M ratio, Spearman's correlation two-tail $r = 0.55$, $p < 0.05$). (B) Similarly, the INCAT score progression index (the increase in the INCAT score per month) had a positive correlation with the P/M ratio (Spearman's correlation two-tail $r = 0.54$, $p < 0.05$).

Please cite this article in press as: Hamada, Y., et al., Binding specificity of anti-HNK-1 IgM M-protein in anti-MAG neuropathy: Possible clinical relevance. Neurosci. Res. (2014), http://dx.doi.org/10.1016/j.neures.2014.09.010

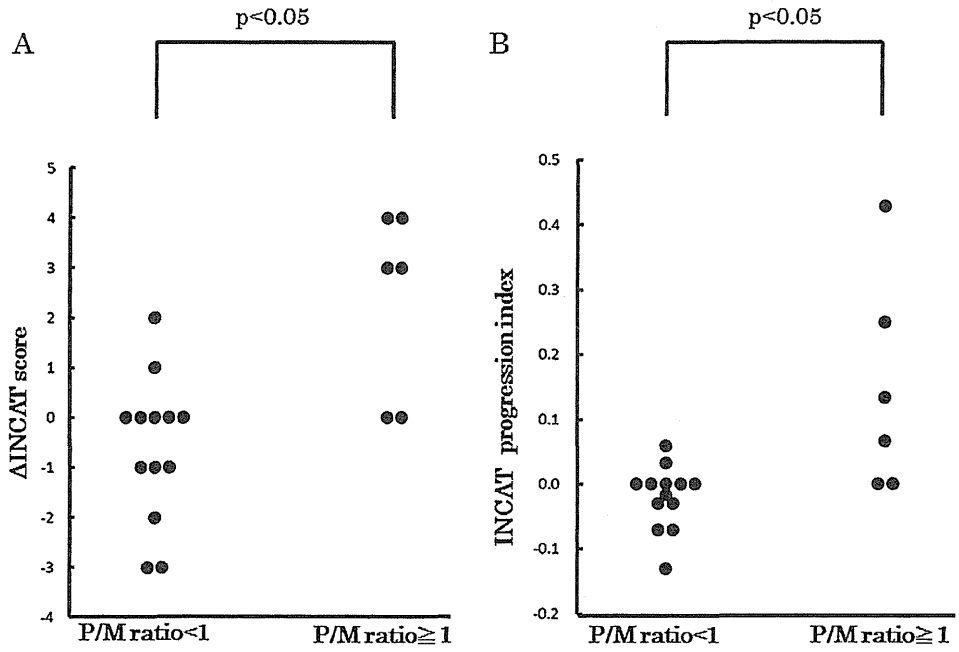

Fig. 3. (A) The ΔINCAT score (the increase in the INCAT score) associated with the ratio of anti-phosphacan to anti-MAG antibody activities (P/M ratio) ≥1 was significantly higher than that with the P/M ratio <1 ($p < 0.05$). (B) The INCAT score progression index (the increase in the INCAT score per month) associated with the P/M ratio ≥1 was significantly higher than that with P/M ratio <1 ($p < 0.05$).

showed that phosphacan is a repulsive substrate for adhesion and neurite regeneration (Sango et al., 2003). So far the role of phosphacan in the peripheral nervous system has not been examined *in vivo*. The observed association of a high P/M ratio with poor prognosis indicates a possible role of immunoreactivity against phosphacan in the pathogenesis of neuropathy, though confirmation must await future studies.

We previously reported variation of the reactivities of IgM M-proteins in sera from 12 patients with neuropathy based on Western blotting using the myelin fraction from human cauda equine and white matter. Of the 12 patients, response of treatment was known in 10. We found that four sera recognized MAG, P0, and PMP-22, while the other six sera only reacted with MAG. The three patients who were responsive to immunotherapy belonged to the latter group (Shiina et al., 2001). Weiss et al. also found that 3 of 11 patients with anti-MAG neuropathy showed a response to IVIg treatment and had no antibody activity to P0 or PMP-22 (Weiss et al., 1999). The variable activities of IgM M-proteins to HNK-1-expressing antigens may therefore underlie the varied response to immunotherapy in IgM paraproteinemic neuropathy. Unfortunately, both studies used human materials with no statistical analyses. Recombinant proteins used in the current study are more useful than human materials, based on availability and reproducibility to increase a sample size for statistical analyses.

In conclusion, we established an ELISA method with recombinant proteins to test the distinct reactivities of antibodies to HNK-1-bearing proteins. Using this method, we found that strong reactivities of anti-MAG antibodies to phosphacan were significantly associated with treatment resistance or progressive clinical courses. The mechanism underlying such association remains unclear, but we speculate that a larger P/M ratio of an anti-MAG antibody reflects a broader range of reactivity against a larger number of HNK-1 bearing antigens in peripheral nerves, resulting in severer neuropathy. Future studies may include a larger-scale analysis to establish the sensitivity and specificity of the method for clinical relevance.

Funding

This work was supported in part by Grants-in-Aid for Scientific Research on Innovative Areas (26110721 [SK], 24110518 [SK] and 23110006 [SO]) and Grants-in-Aid for Scientific Research (B) (24390225 to SK) from MEXT, and in part by the Ministry of Health, Labor and Welfare of Japan (Health and Labour Sciences Research Grant on Comprehensive Research on Disability, Health and Welfare, H21-012 to SK and SO, and on Intractable Diseases, H23-017 to SK).

Author contributions

YH and SK have contributed to the design or conceptualization of the study; YH, MH, MK, MS, KT and SK have contributed to analysis or interpretation of the data; YH has contributed to experiment by ELISA of the study; JM, KY and SO have contributed to product of the antigens; SO and SK have contributed to drafting or revising the manuscript for intellectual content.

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Please cite this article in press as: Hamada, Y., et al., Binding specificity of anti-HNK-1 IgM M-protein in anti-MAG neuropathy: Possible clinical relevance. Neurosci. Res. (2014), http://dx.doi.org/10.1016/j.neures.2014.09.010

RESEARCH PAPER

A multicentre prospective study of Guillain-Barré Syndrome in Japan: a focus on the incidence of subtypes

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▶ Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136/jnnp-2013-306509).

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Received 7 August 2013
Accepted 3 November 2013
Published Online First 22 November 2013

▶ http://dx.doi.org/10.1136/jnnp-2013-306212

To cite: Mitsui Y, Kusunoki S, Arimura K, *et al. J Neurol Neurosurg Psychiatry* 2015;**86**:110–114.

ABSTRACT

Objective Guillain–Barré Syndrome (GBS) is classified into the two major subtypes; acute inflammatory demyelinating polyneuropathy (AIDP) and acute motor axonal neuropathy (AMAN). Previous studies have suggested that AIDP is predominant and AMAN is rare in Western countries, whereas AMAN is not always uncommon in East Asia. We aimed to clarify the incidence of the subtypes of GBS in Japan.

Methods We performed a prospective multicentre survey over 3 years (2007–2010). Clinical and electrophysiological findings were collected from 184 patients with GBS in 23 tertiary neurology institutes. Anti-ganglioside antibodies were measured by ELISA. We also surveyed the incidence of Fisher syndrome (FS).

Results By electrodiagnostic criteria of Ho *et al*, patients were classified as having AIDP (40%), or AMAN (22%), or unclassified (38%). Anti-GM1 IgG antibodies were found for 47% of AMAN patients, and 18% of AIDP patients ($p<0.001$). There were no specific regional trends of the electrodiagnosis and anti-GM1 positivity. During the same study period, 79 patients with FS were identified; the percentage of FS cases out of all cases (FS/(GBS+FS)) was 26%.

Conclusions The frequency of GBS patients with the electrodiagnosis of AMAN by single nerve conduction studies is approximately 20% in Japan, and the AMAN pattern is closely associated with anti-GM1 antibodies. The incidence of FS appears to be much higher in Japan than in Western countries.

INTRODUCTION

The concept of Guillain–Barré syndrome (GBS) changed in the 1990s due to the recognition of acute motor axonal neuropathy (AMAN) as an axonal subtype of GBS.[1–3] Thus, GBS is now divided into two major subtypes: AMAN and acute inflammatory demyelinating polyradiculoneuropathy (AIDP), mainly based on neurophysiological criteria. AMAN has been associated with antecedent *Campylobacter jejuni* infection and autoantibodies to gangliosides, especially to GM1 and GalNAc-GD1a.[4 5] Previous reports have suggested that AIDP is frequent and AMAN is rare in Western countries, whereas AMAN is common in Asia and central and southern America.[2 3 6–18] Some regional studies in Japan[7–9 17] suggested that the frequency of AMAN seems to be higher than in Western countries (23–48%), but this has not been shown in a large nationwide prospective survey. In the current study, we examined the electrophysiological subtypes in a large prospective cohort of the Japanese population and compared these data with those from Western countries and China.

METHODS

Survey procedures

To investigate the incidence of axonal GBS, a nationwide multicentre prospective survey of GBS was conducted by the Research Committees for Neuroimmunological Diseases sponsored by the Ministry of Health, Labor and Welfare, Japan. Data for patients with GBS that fulfilled Asbury and Cornblath[19] criteria were collected from 23 university hospitals or tertiary hospitals in the Japan GBS study group (see online supplementary appendix) between August 2007 and July 2010. In this period, 222 patients with GBS were treated at these hospitals. Of these patients, 184 gave informed consent for utilisation of personal data, storage and assay of biological materials for research purposes. Data for these 184 patients (male 114, female 70, age: 45.5 ±18.5) were collected prospectively and analysed. This is a large multicentre study in Japan.

The number of patients with Fisher syndrome (FS) was also surveyed. FS was diagnosed based on clinical symptoms characterised by acute and self-limited ophthalmoplegia, ataxia and areflexia.[20] The study design was agreed upon and approved by the ethics committee of Kinki University Faculty of Medicine.

Clinical information

The following clinical information was prospectively collected for the 184 patients using predefined format: type of antecedent events (respiratory, gastrointestinal, others and none); GBS disability score[21] from 0 to 6 (at nadir and at 6 months after onset: 0, healthy; 1, minor symptoms or signs, able to run; 2, able to walk 5 m independently; 3, able to walk 5 m with a walker or support; 4, bedbound or chair-bound; 5, requiring assisted ventilation; 6, death); sensory disturbance (yes or no); deep sensory disturbance (yes or no); cranial nerve palsy (yes or no); ophthalmoplegia (yes or no); facial nerve palsy (yes or no); and oropharyngeal palsy (yes or no).

Electrophysiology

An electrophysiological study was performed within 21 days of symptom onset. If two or more studies were performed during the period in the single patients, later results were principally adopted. In the cases that second results classified into unclassified by Ho's criteria or equivocal by Hadden's criteria, first results were adopted to avoid unclassified or equivocal cases.

Motor conduction studies were performed in the median, ulnar and tibial nerves to measure the amplitude and duration of the negative peak of the compound muscle action potential (CMAP) in distal (dCMAP) and proximal (pCMAP) stimulation, conduction velocity (CV), the distal motor latency (DML), and the minimal F-wave latency. These parameters (DML, dCMAP, pCMAP and CV) are expressed as a percentage of the upper (ULN) or lower (LLN) limit of normal for the test at each hospital. Sensory nerve conduction studies were also performed in the median, ulnar, and sural nerves, but these data were not used for classification of subtypes.

Electrophysiological criteria for classification of AIDP and AMAN

Two sets of diagnostic criteria were used to discriminate between demyelination and axonal damage: the criteria of Ho *et al*[2] (referred to as Ho's criteria) were used for comparison with data from northern China, and those of Hadden *et al*[3] (Hadden's criteria) were used for comparison with data from Western countries. In Ho's criteria, patients with equivocal, inexcitable or normal conditions are categorised as 'unclassified'; therefore, patients are classified into three categories of AMAN, AIDP and unclassified in these criteria. In Hadden's criteria, patients are classified into five categories of primary axonal, primary demyelination, equivocal, inexcitable and normal.

Antiganglioside antibodies assay

IgG antibodies against gangliosides GM1 and GQ1b were measured by ELISA performed basically as described before.[22] These serological data were obtained from 174 of 184 patients.

Statistical analysis

Statistical analysis was performed using SPSS V.10 software. Differences in ratios between two groups were tested for significance by χ^2 test, or by Fisher exact test when the criteria for a χ^2 test were not fulfilled. Analyses of numerical variables were initially performed by Kruskal–Wallis H test. When significance was found, a Mann–Whitney U test was used to determine the significance of differences between subgroups.

RESULTS

Electrophysiological categories

In the electrophysiological test, 73 patients (40%) met Ho's criteria for AIDP, 40 (22%) were classified as AMAN, and 71 (38%) were unclassified. Among 40 patients classified into AMAN, five (13%) was defined as acute motor and sensory axonal neuropathy (AMSAN) using electrophysiological criteria.[23] The distribution of these subtypes differed significantly from those in northern China.[2] (figure 1A). In the same test, 83 patients (45%) met Hadden's criteria for demyelination; 24 (13%) were classified as axonal, none as inexcitable, 16 (9%) as normal and 61 (33%) as equivocal. The distribution of these subtypes also differed significantly from those in Western countries reported by Hadden *et al*[3] (figure 1B).

Clinical and electrophysiological profiles

The clinical features of the patients are shown in tables 1 and 2. The 1st electrophysiological studies were performed at 9.2 ±5.3 days from symptom onset. Clinical information on antecedent events, cranial neuropathy, sensory disturbance and GBS disability score at nadir was obtained in all 184 patients, and 118 of 184 patients were also evaluated using the GBS disability score at 6 months after onset of GBS. Antiganglioside antibodies were examined in 174 patients. No patient had vaccination as an antecedent event. Two patients died during the study period, giving a mortality rate of 1.1%, and 27 patients (14.7%) required mechanical ventilation support.

There was no significant difference in the type of antecedent events or GBS disability score at any time between the AMAN

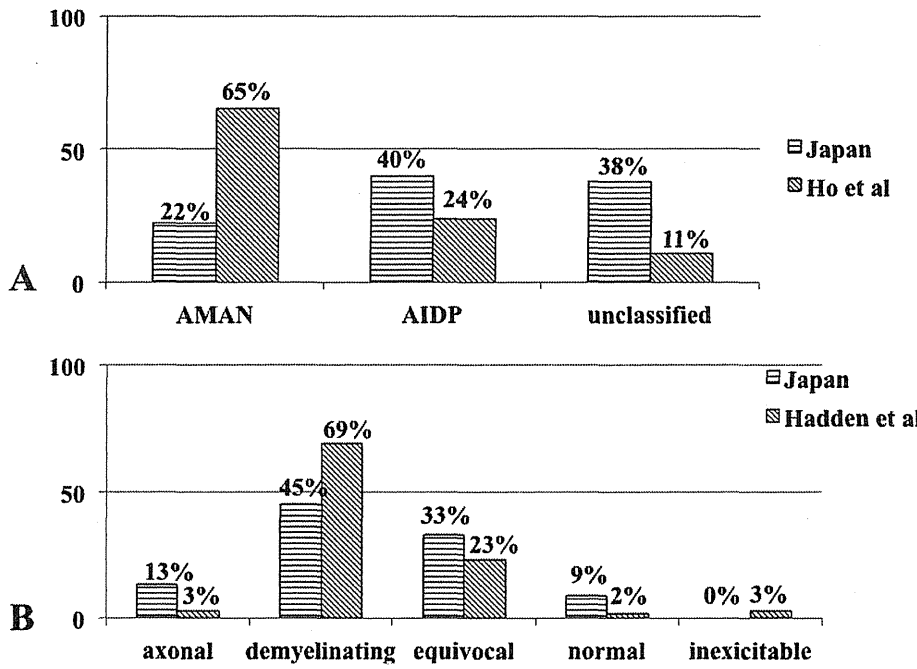

Figure 1 Comparison of data between the present study and previous studies. (A) Japan compared to China (Ho *et al*[1]) (B) Japan compared to Western countries (Hadden *et al*[3]).

Table 1 Clinical features and Hughes functional grade at nadir and 6 month after onset of GBS. Groups are classified by Ho's criteria

Variable	AMAN	AIDP	Unclassified
Age (years)	48.4±20.2	47.7±18.7	41.6±16.8
Gender (male:female)	21:19*	53:20	40:31
Antecedent infection (%)			
Upper respiratory tract	28	29	32
Gastrointestinal	52	41	47
Others	5	4	8
None	15	26	13
Cranial nerve symptoms (%)			
Ophthalmoplegia	15	12	17
Facial nerve palsy	18	36	31
Oropharyngeal palsy	10*	22	31
Overall	30*	51	54
Sensory disturbance (%)	53*	74	59
Deep sensory disturbance (%)	18	23	21
GBS disability score			
At nadir (% of the number of patients more than grade 3 or more)	65	71	63
118 of 184 patients were analysed			
At 6 month after onset (% of the number of patients more than grade three or more)	21	11	12
Antiganglioside antibody (%)	174 of 184 patients were analysed		
GM1-positive	54*	14*	27
GQ1b-positive	9	11	16

*p<0.05 AMAN versus AIDP.
AIDP, acute inflammatory demyelinating polyneuropathy; AMAN, acute motor axonal neuropathy; GBS, Guillain-Barré syndrome.

and AIDP classified by Ho's criteria, however, ratio of male patients, presence of overall cranial neuropathy, bulbar palsy and sensory disturbance were significantly less frequent in AMAN than in AIDP (table 1). There were no significant differences in clinical status except ratio of male patients among the subgroups classified by Hadden's criteria (table 2). An anti-GM1 antibody-positive status was more frequent in patients with AMAN compared to those with AIDP based on Ho's criteria, and also more frequent in those in the axonal subgroup compared to the demyelination subgroup based on Hadden's criteria. An anti-GQ1b antibody-positive status showed no significant difference among subgroups based on Ho's criteria

Table 2 Clinical features and Hughes functional grade at nadir and 6 months after onset of GBS. Groups are classified by Hadden's criteria

Variable	Axonal	Demyelination	Equivocal	Normal
Age (years)	46.4±19.9	48.2±18.0	45.1±18.2	31.6±14.5
Gender (male:female)	16:8*	60:23	33:28	5:11
Antecedent infection (%)				
Upper respiratory tract	17	29	36	31
Gastrointestinal	63	44	39	50
Others	8	4	7	13
None	12	23	18	6
Cranial nerve symptoms (%)				
Ophthalmoplegia	21	13	21	33
Facial nerve palsy	25	38	28	33
Oropharyngeal palsy	25	22	20	38
Overall	33	49	49	50
Sensory disturbance (%)	46	72	61	63
Deep sensory disturbance (%)	8	23	26	13
GBS disability score				
At nadir (% of the number of patients more than grade three or more)	83	70	67	19
118 of 184 patients were analysed				
At 6 month after onset (% of the number of patients more than grade three or more)	21	10	14	0
Antiganglioside antibody (%)	174 of 184 patients were analysed			
GM1-positive	54*	18*	32	27
GQ1b-positive	5	9	14	38

No case was classified as inexcitable.
*p<0.05 axonal versus demyelination.
GBS, Guillain–Barré syndrome.

and Hadden's criteria. There were no specific regional trends of the electrodiagnosis and anti-GM1 positivity in Japan.

Occurrence of FS

A total of 79 patients with FS were identified in 23 hospitals in the Japan GBS study group between August 2007 and July 2010. In the same period, there were 222 patients with GBS; therefore, the percentage of FS cases out of all cases (FS/(GBS +FS)) was 26%.

DISCUSSION

This Japanese multicentre prospective study showed an incidence of AMAN (axonal GBS) of 13–22% in electrophysiological evaluation of patients with early stage GBS. This incidence is significantly lower than that in a Chinese study,[2] but higher than that in Western countries.[3] In order to ensure the precise diagnosis of demyelinating or axonal type of GBS, we selected hospitals having neurologists specialised in electrophysiology. The selected hospitals were located throughout Japan. Therefore, the ratio of subtypes of GBS, demyelinating or axonal in this study, should precisely reflect the situation in Japan.

The incidences of axonal GBS found in various countries worldwide based on electrophysiological criteria are summarised in table 3. All studies classified AMAN and AIDP based on Ho's criteria, Hadden's criteria or both. Some studies included adults and children, while others focused only on children. The reasons for the different incidences of AMAN in different countries are unclear. A regional difference is apparent, since AIDP is more common in Western countries, while AMAN is more frequent in such Asian countries as China[2] and Bangladesh.[14] Interestingly, a report from Israel,[11] which is geographically located between Europe and Asia, showed an intermediate frequency of AMAN. On the other hand, the frequency of AMAN in India,[12] one of the largest countries in Asia, is lower than that in China and Bangladesh, which seems to be inconsistent with the regional trend.

The frequency of preceding *C jejuni* infection has been reported to be higher in Asian countries than in Western countries, and this was thought to be the cause of the higher incidence of the axonal type of GBS in Asia.[2] However, a collaborative study in Japan and The Netherlands[24] showed that the incidence of antecedent *C jejuni* infection in GBS in Japan was not higher than that in The Netherlands. Paradiso *et al*[6] pointed out that 90% of children with AMAN in Argentina are from rural areas without running water, while a recent report from China showed that the incidence of AMAN was lower than that in 1995.[2 18] Environmental factors may differ among districts within the same country, and the status of hygiene may be related to the incidence of AMAN, since the pathogenesis is thought to be correlated with gastrointestinal infection by *C jejuni*. Besides antecedent *C jejuni* infection, genetic factors might also be related to this difference, however, there is insufficient evidence. Taken together, these results suggest that regional differences mainly account for the different incidences of AMAN among various countries, but this may not fully explain this discrepancy.

The method used to classify AIDP and AMAN may be another reason for the different incidences. Ho's and Hadden's criteria were widely used to discriminate AMAN from AIDP in these studies. Hadden's criteria classify the findings of conduction block as demyelination, but Ho's criteria do not do so. Therefore, Hadden's criteria provide a wider definition of AIDP than Ho's criteria, which may partly explain the different incidences among previous studies. Furthermore, Ho's and Hadden's criteria do not take sensory conduction study into consideration. Adding data of sensory conduction study may contribute to more adequate classification of subtypes.

Another point of interest is the difference in clinical features and outcome between AMAN and AIDP. Originally, Feasby *et al*[1] described five patients with the axonal form of GBS who showed a poor outcome. Since then, the axonal form (AMAN) has been believed to have a more severe clinical course than that

Table 3 Previous studies of the incidence of AMAN (or axonal GBS) in various countries

	Year	Region	Age (years) of patients	Criteria for classification	% AMAN or axonal GBS, and number of patients
Ho *et al*[2]	1995	Northern China	All ages, 60% <20	Ho	65 of 129
Hadden *et al*[3]	1998	11 Western countries	All patients >16 years old	Hadden	3 of 369
Paradiso *et al*[6]	1999	Argentina	Children (1–14)	Hadden	30 of 61
Ogawara *et al*[7]	2000	Japan	All ages (3–80)	Ho	33 of 86
Hiraga *et al*[8]	2003	Japan	All ages, no details	Ho	47 of 131
Nagasawa *et al*[9]	2006	Japan	Children (1–15)	Ho	48 of 31
Nachamkin *et al*[10]	2007	Mexico	Children (1–17)	Hadden	48 of 95*
Kushnir *et al*[11]	2007	Israel	All ages (15–84)	Hadden	37 of 40
Kalita *et al*[12]	2008	India	All ages, 12% <13	Ho	14 of 51
Gupta *et al*[13]	2008	India	All ages	Ho	11 of 142
Islam *et al*[14]	2010	Bangladesh	All ages, 73% <30	Hadden	67 of 100
Uncini *et al*[15]	2010	Italy	No information	Ho	18 of 55
				Hadden	18 of 55
Akbayram *et al*[16]	2011	India	Children (0–15)	Ho	31 of 36
Sekiguchi *et al*[17]	2012	Japan	All ages (12–81)	Ho & Hadden	23 of 103
		Italy	All ages (9–79)	Ho & Hadden	17 of 53
Ye *et al*[18]	2013	Northeastern China	All ages (3–75)	Hadden	33 of 99
Present study		Japan	All ages (1–84) 4% <20	Ho	18 of 184
				Hadden	10 of 184

*121 patients enrolled in this study, but electophysiological data were available for only 95 patients.
AMAN, acute motor axonal neuropathy; GBS, Guillain–Barré syndrome.

of AIDP. However, Hadden *et al*[3] found no significant differences between AMAN and AIDP in their series. In our series, there were no significant differences in clinical outcome at any time between AMAN and AIDP either on Hadden's criteria or Ho's criteria. Furthermore, there were no significant differences in clinical features of antecedent events, involvement of the cranial nerve, and sensory disturbance among subtypes classified based on Hadden's criteria; however, oropharyngeal palsy and sensory disturbance were significantly less frequent in AMAN than AIDP on Ho's criteria. Our results also showed significant male predominance in AIDP or demyelinating groups. We could not find the reason for it. As the previous studies had no such gender gap, further investigation may be necessary.

The anti-GM1 antibody-positive rate was significantly higher in patients with AMAN than in those with AIDP. This trend was similar for subgroups classified using Ho's and Hadden's criteria. A large Chinese study[2] and a study in Western countries[3] failed to show a correlation between anti-GM1 antibody and electrodiagnosis of AMAN or axonal GBS, but accumulated clinical and experimental evidence indicates a close relationship among axonal GBS, an anti-GM1 antibody positive status, antecedent gastrointestinal events, and *C jejuni*. Recently, in a study in Italy, it was also reported that an anti-GM1 antibody-positive status was also correlated with AMAN.[15] Our results supported the evidence of correlation between an anti-GM1 antibody-positive status and axonal damage, however, 14% of AIDP patients also had anti-GM1 antibody, indicating that the association is not so rigid. There were no significant differences in GQ1b-positive status between AIDP (demyelinating) and AMAN (axonal) subtypes.

Sekiguchi *et al*[17] suggested that in GBS, clinical and final electrophysiological profiles are better determined by antibodies to such gangliosides as GM1, GD1a, GalNAc-GD1a and GM1b, rather than by electrodiagnosis in the early stage, and that electrodiagnosis at 3–6 weeks after GBS onset differentiates AMAN and AIDP more correct than electrodiagnosis performed in week 1 or 2. As a part of this reason, they also pointed out some patients with AIDP on the initial electrophysiological studies turned to AMAN or unclassified because the slowing of distal latency was rapidly recovered. This issue should be investigated in future studies.

In this cohort study, we also surveyed cases of FS and found that FS accounted for 26% of all GBS+FS cases. A previous study in an Italian cohort found that FS accounted for only 3% of all GBS+FS cases,[25] whereas reports from a single hospital in Taiwan found this value to be 18–19%.[26 27] In Japan, a study of 50 consecutive cases with FS indicated a value of 34%.[28] These limited results suggest that FS might have a higher incidence in East Asia than in Europe.

Contributions YM: drafting/revising the manuscript, analysis or interpretation of data, acquisition of data. SK: study concept or design, study supervision, obtaining funding. KA: drafting/revising the manuscript, acquisition of data. RK: drafting/revising the manuscript, acquisition of data. TK: drafting/revising the manuscript, acquisition of data. SK: drafting/revising the manuscript, acquisition of data. MS: drafting/revising the manuscript, acquisition of data. KT: and GBS epidemiological study group of Japan: acquisition of data.

Funding Supported in part by a Health and Labour Sciences Research Grant on Intractable Diseases (H23-Nanchi-Ippan-017) from the Ministry of Health, Labour, and Welfare, Japan, and a Scientific Research B Grant (No. 24390225) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

Competing interests None.

Patient consent Obtained.

Ethics approval the Ethics Committee of Kinki University Faculty of Medicine.

Provenance and peer review Not commissioned; externally peer reviewed.

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A multicentre prospective study of Guillain-Barré Syndrome in Japan: a focus on the incidence of subtypes

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J Neurol Neurosurg Psychiatry 2015 86: 110-114 originally published online November 22, 2013
doi: 10.1136/jnnp-2013-306509

ARE MULTIFOCAL MOTOR NEUROPATHY PATIENTS UNDERDIAGNOSED? AN EPIDEMIOLOGICAL SURVEY IN JAPAN

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Accepted 4 June 2013

ABSTRACT: *Introduction:* Our objective was to do an epidemiologic survey of patients with multifocal motor neuropathy (MMN) in comparison with those with amyotrophic lateral sclerosis (ALS) in Japan. *Methods* In this retrospective study, we examined 46 patients with MMN and 1,051 patients with ALS from major neuromuscular centers in Japan from 2005 to 2009. Diagnosis was based on the European Federation of Neurological Societies/Peripheral Nerve Society (EFNS/PNS) and the revised El Escorial criteria. The efficacy of intravenous immunoglobulin (IVIg) was also taken into consideration in the diagnosis of MMN. *Results* The ratio of MMN to ALS patients (0–0.10) varied among the centers, but mostly converged to 0.05. The prevalence was estimated to be 0.29 MMN patients and 6.63 ALS patients per 100,000 population. *Conclusions* The frequency of MMN patients was around 1 out of 20 ALS patients, and MMN was possibly underdiagnosed in some centers.

Muscle Nerve **000**:000–000, 2013

Multifocal motor neuropathy (MMN) is characterized by predominant involvement of motor nerves presenting with slowly progressive muscle atrophy and weakness, a typical age of onset between the third and fifth decades of life, and a high prevalence in men.[1–3] The characteristic diagnostic features of MMN are conduction block (CB) in multiple peripheral nerves and the presence of anti-GM1 IgM antibodies.[4–7] However, the diagnosis of MMN may be missed in those without overt evidence of CB or elevated anti-GM1 IgM antibody levels.[8–10] CB may not be detected in MMN patients whose demyelinating lesion is located in proximal nerve segments (e.g., plexus, nerve root)[11] or when it is associated with significant secondary axonal loss.[8,12] Several diagnostic criteria for MMN have been proposed.[13–15] The European Federation of Neurological Societies/Peripheral Nerve Society (EFNS/PNS) criteria may have limited sensitivity due to the possibility of undetected CB.[11,13,16] MMN is treatable with various immunomodulatory therapies, particularly intravenous immunoglobulin (IVIg), and the response may be a feature that distinguishes MMN from lower motor neuron diseases, including amyotrophic lateral sclerosis (ALS).[17,18]

Except for 1 clinic-based study that estimated the prevalence of MMN to be approximately 10% of that of ALS,[19] detailed large-scale epidemiological studies of MMN have been undertaken rarely. The lack of knowledge of the above technical limitations in the diagnosis of MMN, and the rarity of the disease, might lead clinicians to underdiagnose MMN. We therefore conducted an epidemiological survey of MMN in major neuromuscular centers in Japan and compared it with ALS, whose prevalence is known.

A brief preliminary report of this study has been published in Japanese.[20]

MATERIALS AND METHODS

Patients. This study was based on a retrospective hospital-based survey in Japan. The diagnosis of MMN was based on the 2006 EFNS/PNS criteria and the response to IVIg. The 2006 criteria were used, because the latest criteria were not available at the time of clinical evaluation. We excluded patients with MMN who died during the course of the study because one of those patients might actually have had ALS. The diagnosis of ALS was

Abbreviations: ADCB, activity-dependent conduction block; ALS, amyotrophic lateral sclerosis; APB, abductor pollicis brevis; CB, conduction block; CI, confidence interval; CMAP, compound muscle action potential; EFNS/PNS, European Federation of Neurological Societies/Peripheral Nerve Society; IVIg, intravenous immunoglobulin; MMN, multifocal motor neuropathy

Key words: amyotrophic lateral sclerosis; conduction block; diagnosis; multifocal motor neuropathy; prevalence

The authors report no disclosures.

This research was partially supported by Japan Society for the Promotion of Science Grantn-id for Young Scientists (B), 23790995, 2012. This work was also supported by grants from the Neuroimmunological Disease Research Committee and the Ministry of Health, Labour and Welfare, Japan.

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Published online 00 Month 2013 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/mus.23930

made by using the revised El Escorial criteria, and patients who fulfilled the "clinically definite," "clinically probable," or "laboratory-supported probable" criteria were included for further epidemiological analysis.[21] We excluded those with the "possible" criterion, because it might include MMN and other neuromuscular conditions. First, we sent questionnaires to 46 major neuromuscular centers in Japan requesting clinical information on patients with MMN and ALS at each site. Twenty-five centers (54.3%) expressed their willingness to cooperate in the survey, but 5 centers did not follow through. Of the 20 centers that responded to the second survey, 1 was excluded because of insufficient data for analysis. We further analyzed the clinical records of patients with MMN and ALS from 2005 to 2009. We also checked the results of electrophysiological studies for MMN. The participating 19 centers were not biased geographically and were located throughout Japan.

Electrophysiological Study. Electrophysiological studies were performed at each center using conventional techniques. CB was defined as a >50% reduction in compound muscle action potential (CMAP) amplitude/area from distal to proximal stimulation (i.e., median, ulnar, radial, and deep fibular nerves).[14,22] Other electrophysiological features of peripheral nerve demyelination included reduced motor conduction velocity (motor conduction velocity; <75% of the lower limit of normal), prolonged distal motor latency, or prolonged minimal F-wave latency (>130% of the upper limit of normal), and activity-dependent conduction block (ADCB).[9,22,23] To detect ADCB, CMAPs from the abductor pollicis brevis (APB) were determined with magnetic stimulation of low-cervical nerve roots. One-minute voluntary maximal exercise of the APB was performed, and CMAPs were compared before and after exercise. ADCB was defined by a >50% drop in the CMAP amplitude after exercise.[9]

Epidemiological Study. The Japanese Ministry of Health, Labour, and Welfare has a nationwide registry system for ALS patients, and the number of patients registered in 2009 was 8,492. First, we calculated the ratio of MMN to ALS patients in each center and in the whole study cohort. The number of patients with MMN in 2009 was then estimated based on the ratio of MMN to ALS patients. The population of Japan was based on data from the national population census in 2009. We estimated the prevalence of MMN and ALS (number of cases/100,000 persons), and the 95% confidence interval (CI) in 2009 by assuming a binomial distribution. The number of patients with MMN in Japan (Z) was estimated by $X \times Y/Ny$, where X denotes the total number of patients with ALS in Japan, and Y and Ny denote the number of patients with MMN and ALS, respectively, registered at 19 neuromuscular centers. The variance of Z (Vz) was estimated by the following equation, assuming that X and Y are independent: $Vz = [E(Y) \times E(Y) \times Vx + E(X) \times E(X) \times Vy + Vx \times Vy)/(Ny \times Ny)]$, where E(X) and E(Y) denote the expected value (mean) of X and Y, respectively, and Vx and Vy denote the variance of X and Y, respectively.

We conducted the McNemar test to determine whether the prevalence differed significantly between MMN and ALS. Two epidemiologists (SH and KA) conducted the overall analysis.

Table 1. Demographic data of registered patients.

Characteristic	MMN (n = 46)	ALS (n = 1051)	P value
Onset age, range (mean ± SD), y	16–74 (42.5 ± 15.0)	33–87 (62.2 ± 36.5)	<0.001
Proportion of men (%)	71.7	60.4	0.12

Clinical Characteristics of MMN and ALS. Basic data, such as gender, age of onset, and diagnosis, were collected from patients with MMN and ALS.

Standard Protocol Approval, Registration, and Patient Consent. All investigations were approved by the institutional ethics committee of the University of Tokushima.

Statistical Analysis. The acquired data were processed and analyzed using SPSS software (version 11.0, SPSS Inc., Chicago Illinois). Differences in patient characteristics between MMN and ALS were tested using the Mann-Whitney U-test. Two-tailed P values < 0.05 were considered significant.

RESULTS

Forty-six patients with MMN and 1,051 patients with ALS were analyzed (Table 1). The onset age of MMN was younger (mean, 42.5 ± 15.0 years; range, 16–74 years) than that of ALS (mean, 62.2 ± 36.5 years; range, 33–87 years) ($P < 0.001$; Table 1; Fig. 1). There was no significant difference in the male:female ratio between MMN (71.7%) and ALS (60.4%) ($P = 0.12$).

The ratio of MMN to ALS patients (range: 0–0.10; average: 0.044) varied among centers (Table 2; Fig. 2). There were no MMN patients in 3 centers in the past 5 years (centers A–C). On the other hand, 4 centers showed ratios of approximately 0.10. These centers were not close geographically (centers P–S) and were staffed by board-certified electromyographers with more than

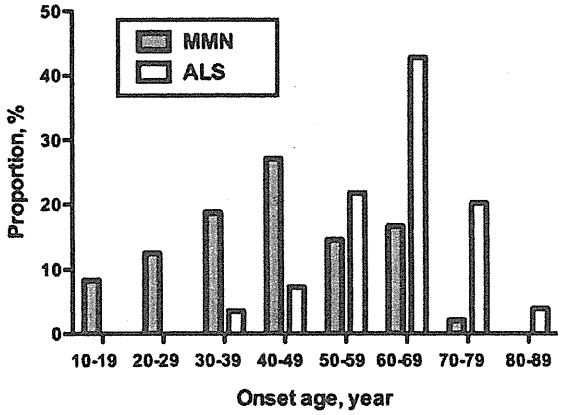

FIGURE 1. Distribution of age of onset. Proportion is defined by the ratio of the number of patients according to age of onset to the total number of MMN or ALS patients. MMN, multifocal motor neuropathy; ALS, amyotrophic lateral sclerosis.

10 years' experience in nerve conduction studies. Some centers had a large number of ALS patients compared with the number of MMN patients (e.g., centers D, E, and M). The number of ALS patients in the national registry in 2009 was 8,492. Based on the ratio of MMN to ALS patients reported in the whole survey (0.044), the number of MMN patients in Japan was estimated to be 372 (95% CI = 266–477). Overall, the prevalence of MMN in Japan was estimated to be 0.29 patients per 100,000 population (95% CI = 0.21–0.37), whereas that of ALS was 6.63 patients per 100,000 population (95% CI = 6.49–6.77) ($P < 0.001$).

DISCUSSION

We conducted an epidemiological survey of patients from multiple neuromuscular centers throughout Japan. The estimated prevalence of MMN in Japan was 0.29 patients per 100,000 population or approximately 1/20 that of ALS. The gender distribution was similar to that reported previously.[19,24] The mean age of onset of MMN was slightly older than that of a previous study.[19] The prevalence in our study was lower than those in prior studies conducted in Europe.[19,25] One clinic-based study in Italy estimated the prevalence of MMN to be approximately 10% that of ALS.[19] A study in the Netherlands reported the prevalence of MMN to be 0.6 patients per 100,000 population.[25] The exact reason for the difference is unknown. Given the similar prevalence of ALS worldwide, a possibility why MMN is less common in Japan would be that it is underdiagnosed, particularly in the centers that showed very low

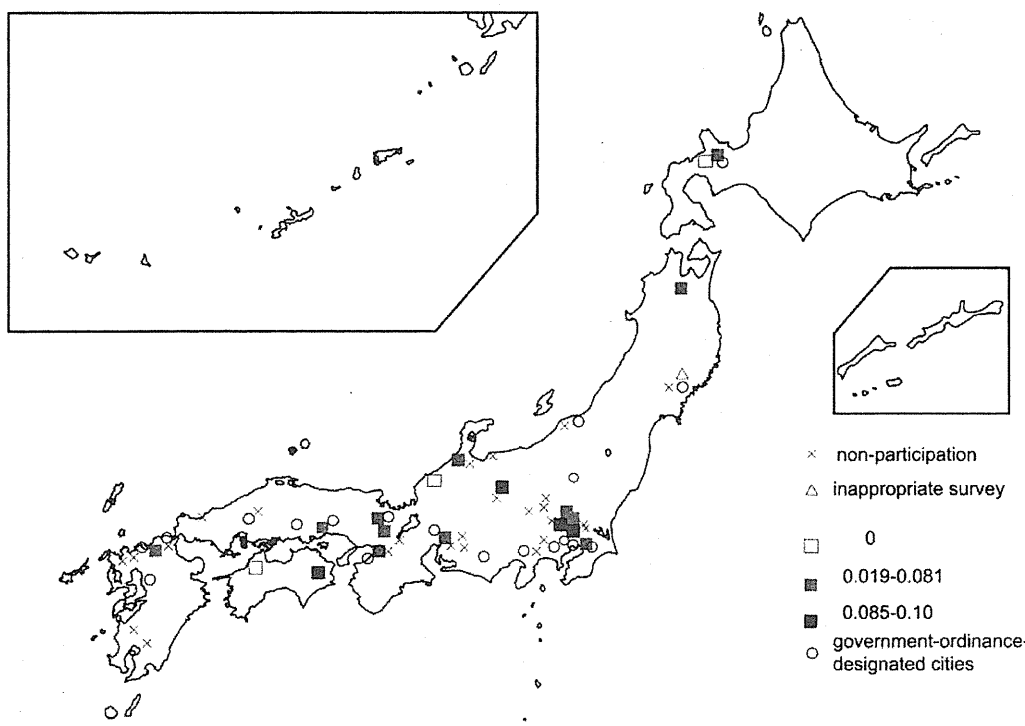

FIGURE 2. Geographical distribution of the ratios of MMN to ALS patients among centers. Crosses: nonparticipating centers; triangle: center that provided inappropriate data; squares: participating centers (open squares: 0; gray squares: 0.019–0.081; black squares: 0.085–0.10). Open circles indicate the highly-populated cities (government-ordinance-designated cities with populations exceeding 0.7 million). MMN, multifocal motor neuropathy; ALS, amyotrophic lateral sclerosis.

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Table 2. Ratios and estimated numbers of patients.

	MMN	ALS	Ratio
Center	*n* [age,* proportion of men (%)]	*n* [age,* proportion of men (%)]	MMN/ALS
A	0	58 (60.0 ± 13.6, 53.4)	0
B	0	26 (68.2 ± 7.2, 53.8)	0
C	0	26 (60.9 ± 12.0, 61.5)	0
D	3 (41.3 ± 7.59, 66.7)	154 (62.8 ± 1.10, 62.3)	0.019
E	2 (57.0 ± 16.0, 50.0)	101 (56.5 ± 12.6, 62.3)	0.020
F	1 (24.0, 100)	43 (63.5 ± 9.41, 60.5)	0.023
G	1 (30.0, 100)	34 (63.2 ± 13.4, 50)	0.029
H	2 (42.0 ± 1.00, 50.0)	58 (62.5 ± 9.40, 41.4)	0.034
I	1 (62.0, 100)	28 (60.5 ± 11.0, 64.3)	0.036
J	1 (23.0, 100)	28 (59.2 ± 12.2, 46.4)	0.036
K	4 (43.0 ± 17.0, 75.0)	94 (64.6 ± 9.42, 54.3)	0.043
L	1 (37.0, 100)	18 (56.2 ± 12.1, 38.9)	0.056
M	2 (45.5 ± 0.50, 50.0)	36 (64.3 ± 9.67, 66.7)	0.056
N	7 (36.0 ± 15.3, 71.4)	113 (64.1 ± 10.4, 58.4)	0.062
O	3 (38.0 ± 8.52, 100)	37 (58.8 ± 12.9, 73.0)	0.081
P	3 (51.3 ± 16.4, 33.3)	35 (63.9 ± 11.6, 65.7)	0.086
Q	9 (42.8 ± 16.3, 77.8)	99 (61.0 ± 11.4, 57.6)	0.091
R	4 (55.3 ± 8.93, 75.0)	43 (67.8 ± 10.6, 33.0)	0.093
S	2 (34.0 ± 17.0, 100)	20 (67.6 ± 8.74, 50.0)	0.10
total†	46	1,051	0.044
Japan	372‡	8,492§	0.044

**Age range: range of onset age (mean ± SD).*

†total: the total number of patients in the 19 centers (A-S).

‡Number of MMN patients in Japan was estimated on the basis of the number of ALS patients (8,492) and the ratio (0.044).

§Number of ALS patients was obtained from the national registry in 2009.

prevalence. Another possibility is that the patients visiting neuromuscular centers were skewed to the elderly population in Japan, and this might have contributed to the lower estimate of MMN than that of ALS. We compared the ratios of MMN with ALS patients among the centers and found considerable variation (0–0.10). The ratios were around 0.10 in the top 4 centers that were widely distributed in Japan and staffed by electromyography experts. It is therefore unlikely that the prevalence of MMN is higher in some parts of Japan that it is in others. Interestingly, 2 of the top 4 centers adopted activity-dependent CB as a criterion for diagnosis of proximal CB. Although activity-dependent CB was not widely performed and might not be observed in some patients with MMN,[26] it appeared to increase the diagnostic sensitivity in this study.

Our study has a few limitations. One of the reasons why some cases of MMN were underdiagnosed is that MMN was often misdiagnosed as other motor neuron diseases, such as ALS. Another reason is that only 19 of the 46 centers provided data for the study. The 46 centers include various neurological facilities that treat neurologic subspecialty or general neurological ones. The low response rate means that we did not intentionally select neurological centers in the first survey. Further diagnostic tests, such as imaging, would further increase the prevalence of MMN, and such efforts would enable us to provide the appropriate immunological treatment. Although MMN is considered to be rare, its accurate diagnosis should rely heavily on clinical suspicion and electrodiagnostic investigations.

We thank the many neurologists (Dr. Shu-ichi Ikeda, Dr. Seiji Kikuchi, Dr. Jun-ichi Kira, Dr. Yuko Shimizu, Dr. Norihiro Suzuki, Dr. Gen Sobue, Dr. Masami Tanaka, Dr. Hidehiro Mizusawa, Dr. Hiroaki Yoshikawa, Dr. Tetsuro Miki, and Dr. Makoto Yoneda) who cooperated in the survey.

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RESEARCH PAPER

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

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Received 14 March 2013
Revised 20 July 2013
Accepted 22 July 2013
Published Online First 7 August 2013

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.

Linked

▶ http://dx.doi.org/10.1136/jnnp-2013-305668

To cite: Shimizu F, Omoto M, Sano Y, *et al. J Neurol Neurosurg Psychiatry* 2014;**85**:526–537.

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

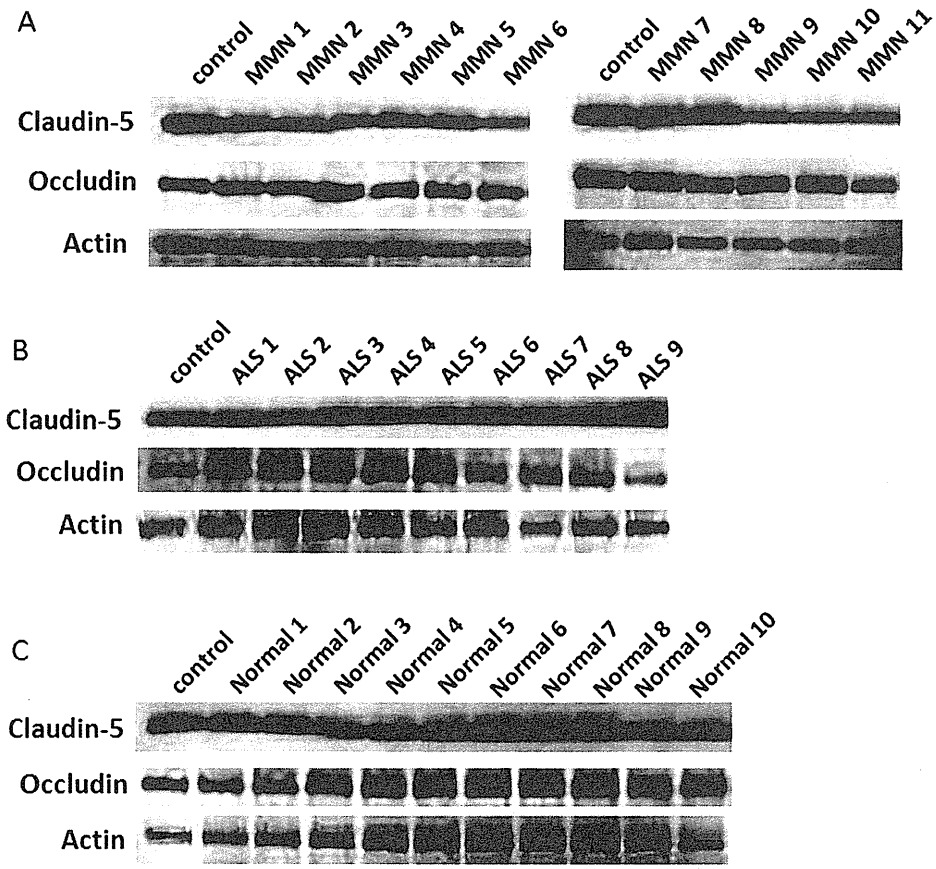

Figure 1 (A–C) The effects of the sera of patient with multifocal motor neuropathy (MMN) on the tight junction proteins in human peripheral nerve microvascular endothelial cells (PnMECs) as determined by a Western blot analysis. The changes of claudin-5 and occludin in PnMECs were determined after exposure 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

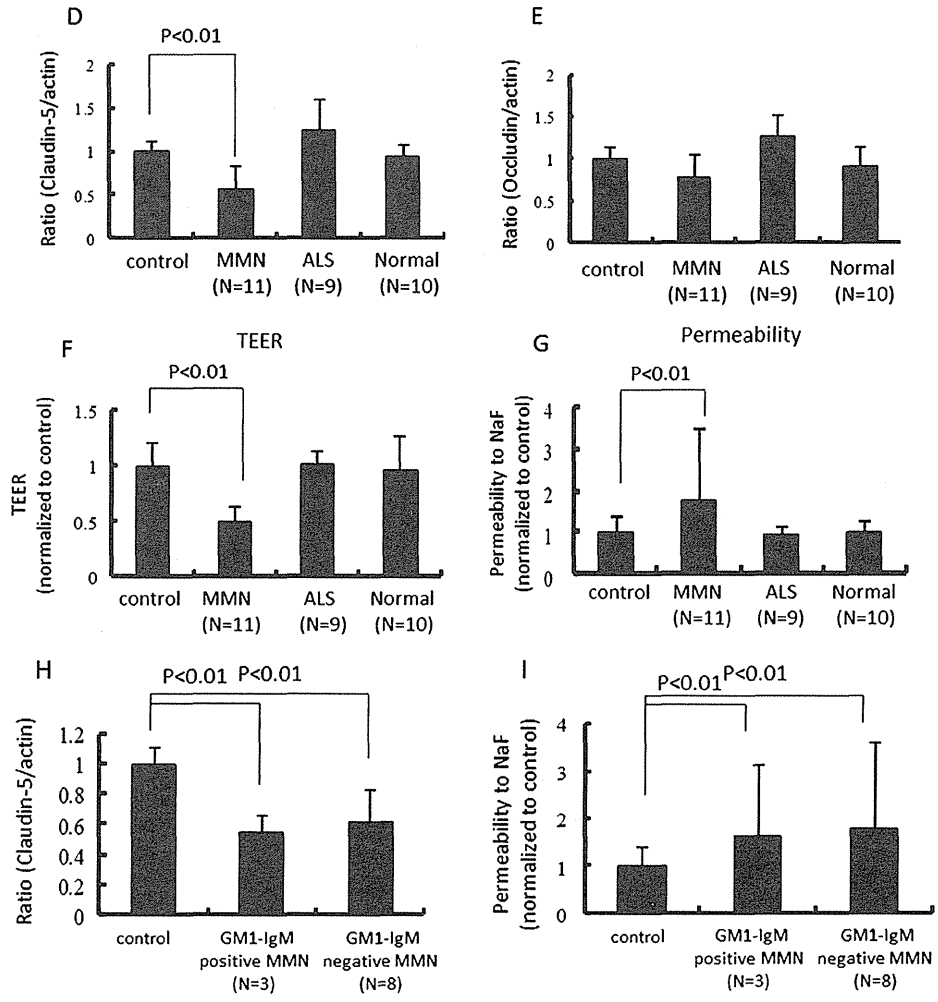

Figure 1 Continued.

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

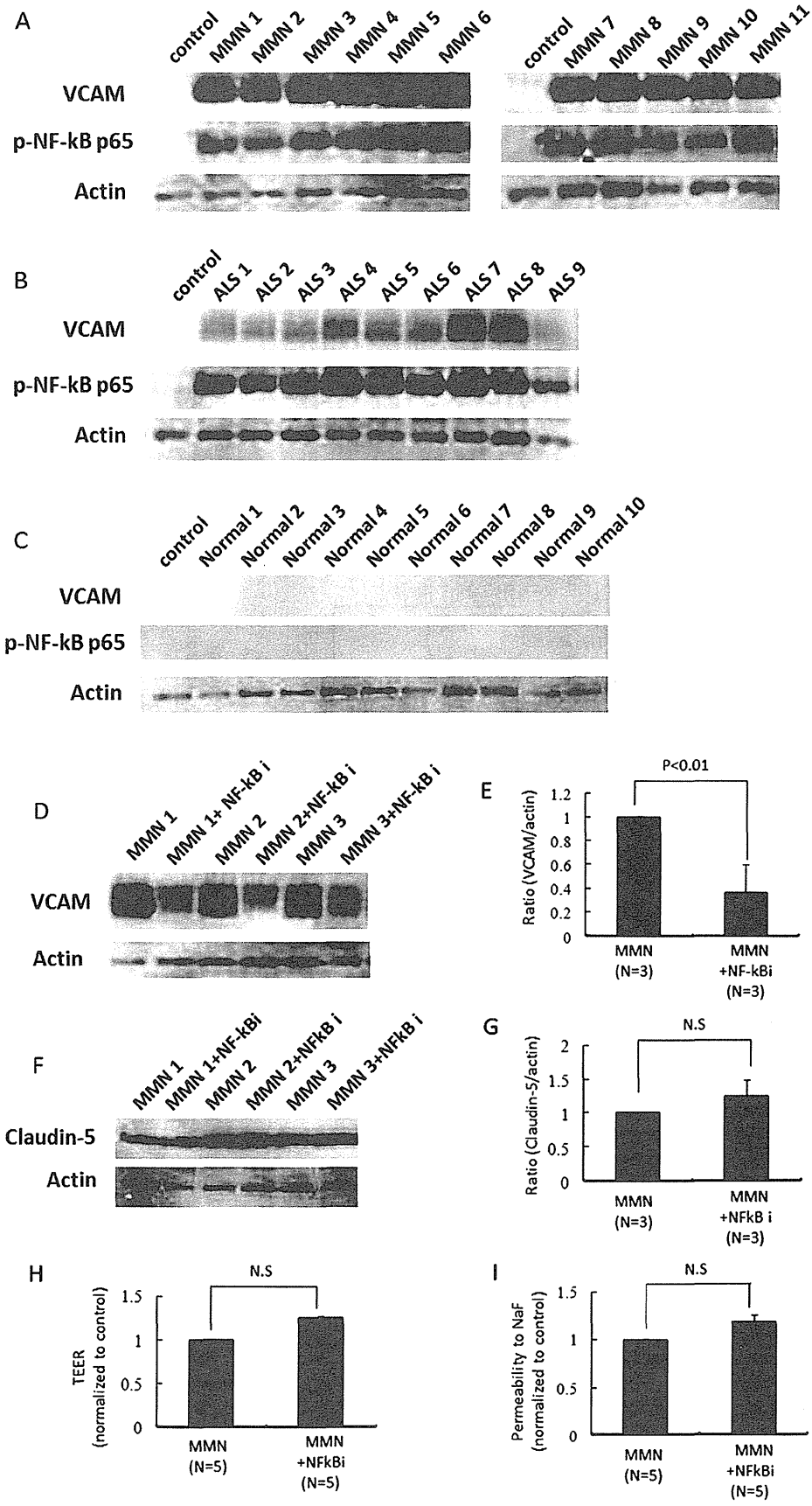

Figure 2 (A–C) The effects of sera on the amount of adhesion molecules in human peripheral nerve microvascular endothelial cells (PnMECs) were determined by a Western blot analysis. The changes in the amount of VCAM-1 protein in PnMECs were determined after exposure to the sera from patients with multifocal 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.