by subcutaneous fat deposits that are isolated by liposuction, they are expected to be a practical cell source for cell-based therapy.

In 2007, Terenghi's group reported Schwann cell differentiation from ADSCs [Kingham et al., 2007]. They obtained Schwann cells by treating ADSCs with β-mercaptoethanol and retinoic acid, followed by a mixture of bFGF, PDGF, forskolin and glial growth factor-2 (also called neuregulin or heregulin), the same method as first reported by Dezawa et al. [2001]. Razavi et al. [2012] also confirmed that the same protocol could successfully induce ADSCs into Schwann cell-like cells (ADSC-Schwann cells). The differentiated cells expressed Schwann cell markers. Coculture of neuronal cells with ADSC-Schwann cells induced neurite outgrowth, suggesting that ADSC-Schwann cells have the ability to elicit neurite extension [Mahay et al., 2008; Brohlin et al., 2009; Faroni et al., 2011]. Radtke et al. [2009] demonstrated that culturing ADSCs in a neurosphere culture followed by dissociation of the formed spheres and the removal of mitogens resulted in the differentiation of ADSCs into Schwann cell-like cells that expressed p75, S-100 and GFAP [Radtke et al., 2009]. Kaewkhaw et al. [2011] reproduced these data and further demonstrated that perinephric ADSCs have a higher potential to be induced into Schwann cells compared with ADSCs from other sources, such as subcutaneous or epididymal fat tissues.

Schwann Cells Induced from MSCs Are Effective for Axonal Regeneration and Functional Recovery in Spinal Cord Injury

Kamada et al. [2005] were the first to show the effectiveness of BMSC-Schwann cells in a completely transected rat spinal cord injury model. The model was created by removing whole T_7 spinal cord segments and replacing them with an artificial tube filled with a mixture of Matrigel and 2×10^6 BMSC-Schwann cells. After 6 weeks, the transected spinal cord was completely connected and nerve fibers positive for tyrosine hydroxylase as well as for serotonin and, to a lesser extent, calcitonin gene-related peptide were detected in the artificial tube (fig. 2a, b). Because whole T_7 spinal segments were completely removed in this case, all of these nerve fibers in the tube were considered to be regenerated axons. BMSC-Schwann cells in the tube maintained their specific Schwann cell type, expressed P0, p75, S-100 and ensheathed axons. Along with histologic analysis, the BBB (Basso, Beattie, Bresnahan) locomotor scale (score of 21 for normal animals) revealed significant recovery of hind limb function (the mean score at 6 weeks was 7, range 5–10), which indicates that all three hind limb joints had extensive movement (fig. 2c). Retransection of the graft at 6 weeks, however, completely abolished the recovered function. The score immediately dropped to 1 after retransection and never recovered (fig. 2d). This fact excluded the possibility that transplanted BMSC-Schwann cells enhanced the activity of a locomotor pattern generator in the spinal cord, and emphasized the direct contribution of BMSC-Schwann cells to functional and histologic recovery in spinal cord injury [Kamada et al., 2005].

Someya et al. [2008] and Kamada et al. [2011] also applied BMSC-Schwann cells to contusion injuries of the rat spinal cord by direct injection of the cells into the crushed site. In these studies, BMSCs and BMSC-Schwann cells were compared, with the BMSC-Schwann cells superior for reducing the volume of the cystic cavity and increasing the number of regenerating axons and functional recovery.

Zaminy et al. [2013] transplanted ADSC-Schwann cells into a rat spinal cord injury model. The cells were loaded into collagen scaffolds and transplanted into 3-mm lesions at T_9–T_{10}. The rats exhibited significantly higher locomotor and sensory scores.

Mechanisms of Schwann Cell Induction from MSCs

The method used to induce Schwann cells from MSCs is the application of β-mercaptoethanol for 24 h, then retinoic acid for 3 days and finally a mixture of bFGF, forskolin, PDGF and neuregulin for 4–5 days, which appears to be applicable to all BMSCs, UC-MSCs and ADSCs. The event occurring in the MSCs during the induction procedure was analyzed in UC-MSCs by Matsuse et al. [2010].

The gene expression pattern showed that UC-MSCs initially do not express P0 and S-100B, but express Sox10 and Krox20 at very low levels [Matsuse et al., 2010] (fig. 3). Stimulation of UC-MSCs with β-mercaptoethanol for 24 h substantially upregulated Krox20, while P0 and S-100B remained negative. Cells further stimulated with retinoic acid newly expressed Sox10. After treatment with a cytokine cocktail of bFGF, forskolin, PDGF and neuregulin, UC-MSCs newly expressed S-100B and P0, and the gene expression levels of Sox10 and Kronx20 increased (fig. 3). In contrast to these expression patterns, a cell marker for the immature neural lineage Hath1 was initially positive in UC-MSCs, but became suppressed after the induction and very faint after treatment of the cells with the cytokine cocktail, which was

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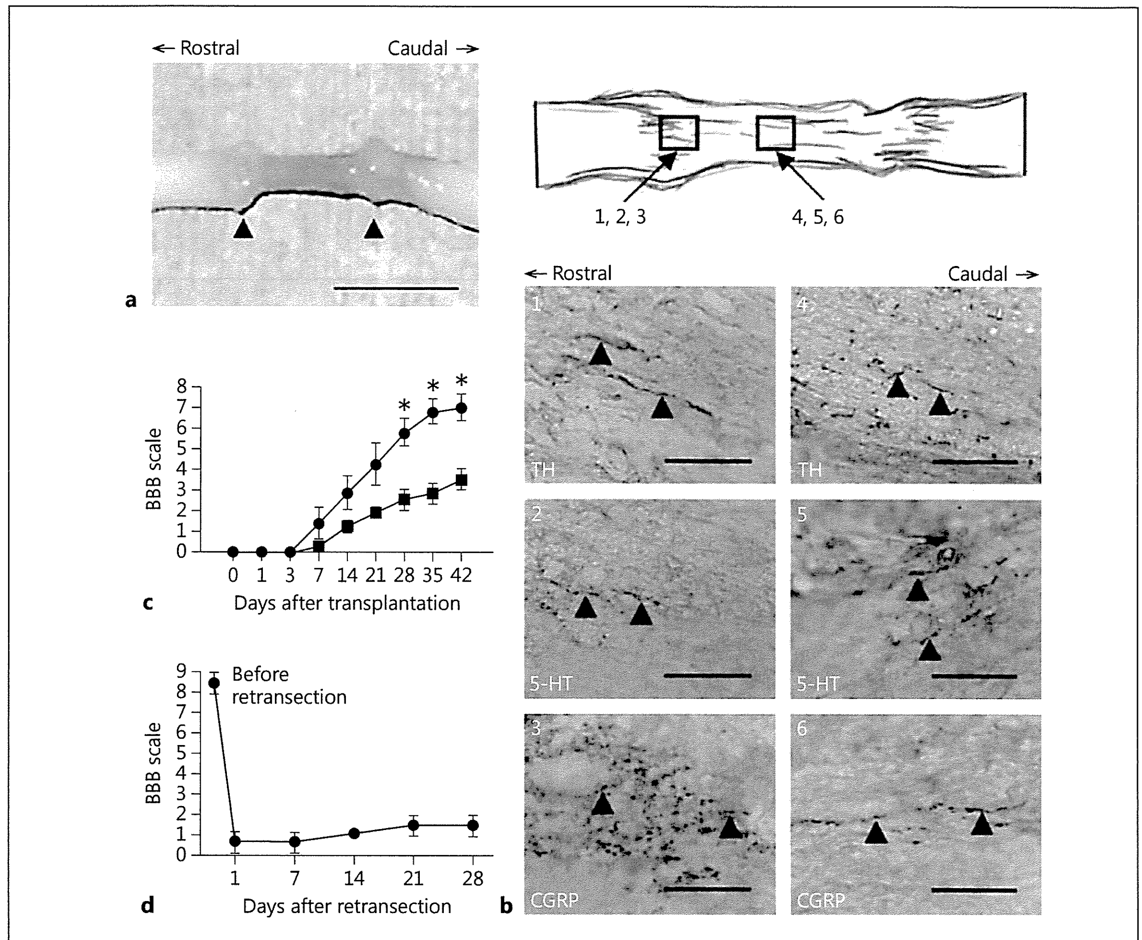

Fig. 2. Rat BMSC-Schwann cells transplanted into a rat spinal cord injury model. **a** An adult rat T_7 spinal cord segment was transected, removed and replaced with an artificial tube filled with rat BMSC-Schwann cells mixed with Matrigel. After 6 weeks the transected spinal cord was completely connected. **b** In the connected region, TH-, 5-HT- and CGRP-positive nerve fibers were observed. **c** The BBB score after transplantation demonstrated substantial recovery in the BMSC-Schwann cell-transplanted group (●) compared to the Matrigel-only group (■). * $p < 0.01$. **d** Retransection of the BMSC-Schwann cell group spinal cord at T_7 level (at 6 weeks) completely abolished the recovered hind limb function and no significant recovery could be observed for 4 weeks. Pictures are reproduced from Kamada et al. [2005]. Scale bars: 5 mm (**a**), 100 μm (**b**).

the final step (fig. 3). These results together suggested that UC-Schwann cells undergo sequential differentiation through this induction process, and the gene expression pattern in UC-MSCs at the final stage of induction becomes almost the same as that in authentic human Schwann cells.

Exposure of UC-MSCs to β-mercaptoethanol and retinoic acid prior to stimulation with the cytokine cocktail is a prerequisite because elimination of these steps fails to induce UC-MSCs into Schwann cells [Matsuse et al., 2010]. β-Mercaptoethanol acts as a reducing agent on MSCs to promote differentiation into neural-lineage cells by the synthesis of glutathione [Hung et al., 2002; Neshati et al., 2010]. Retinoic acid is a well-known factor that acts as a morphogen during development to regulate the expression of various transcription factors that are crucial for early neural determination, such as MASH1 and NeuroD, and has a role in the acquisition of the responsiveness to neurotrophins [Johnson et al., 1992; von Holst et al., 1995].

bFGF functions as a mitogen and accelerates the formation of Schwann cell precursors during Schwann cell transition [Chaudhary and Avioli, 1997; Jessen and Mir-

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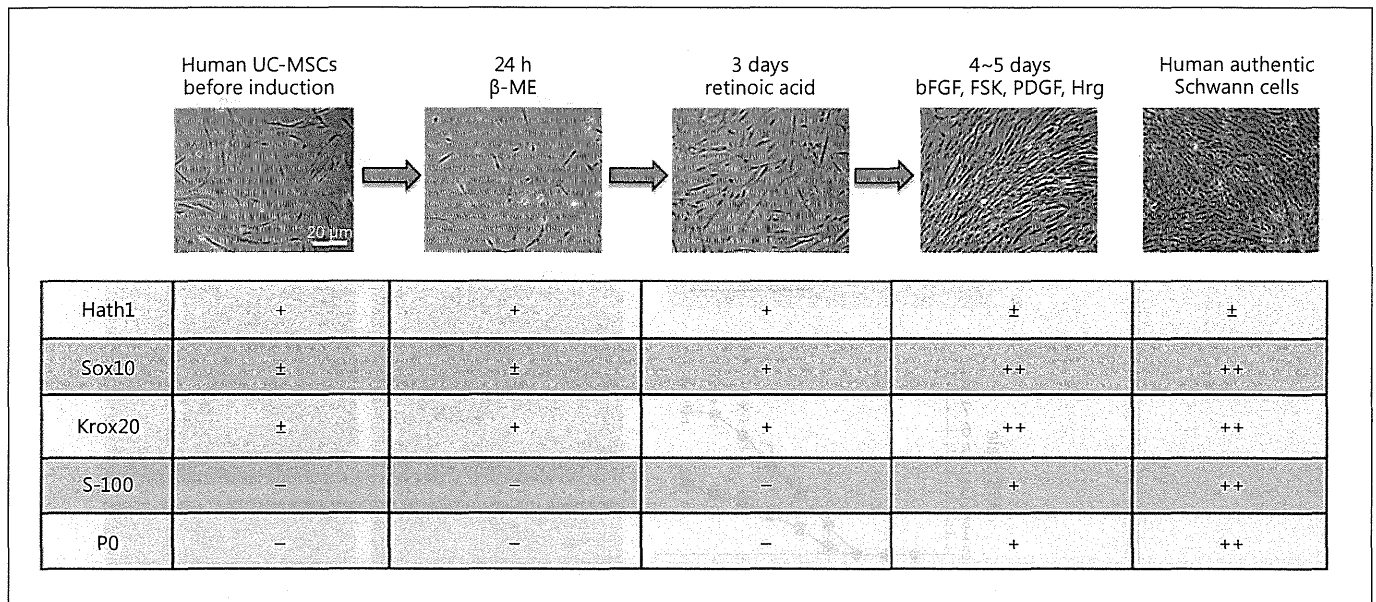

	Human UC-MSCs before induction	24 h β-ME	3 days retinoic acid	4~5 days bFGF, FSK, PDGF, Hrg	Human authentic Schwann cells
Hath1	+	+	+	±	±
Sox10	±	±	+	++	++
Krox20	±	+	+	++	++
S-100	–	–	–	+	++
P0	–	–	–	+	++

Fig. 3. Schwann cell induction from human UC-MSC. The induction procedure, morphological changes in UC-MSCs and expression of factors related to Schwann cells are summarized. Pictures are reproduced from Matsuse et al. [2010]. β-ME = β-Mercaptoethanol; Hrg = heregulin.

sky, 2005] and is reported to be a key factor that induces MSCs into the Schwann cell phenotype [Zhu et al., 2014]. PDGF contributes to DNA synthesis and acts as a mitogen in Schwann cells [Davis and Stroobant, 1990]. Neuregulin, which is also called heregulin or glial growth factor-2, selectively induces Schwann cells from neural crest cells and promotes the survival and proliferation of Schwann cell progenitors [Jessen and Mirsky, 2005]. Forskolin increases the level of intracellular cAMP and the expression level of growth factor receptors [Meyer-Franke et al., 1998]. Therefore, the addition of forskolin to the combination of bFGF, PDGF and heregulin might enhance cellular responses to trophic factors, leading to efficient trophic factor stimulation for Schwann cell differentiation.

A potential underlying mechanism of the Schwann cell induction system would be the combined actions of β-mercaptoethanol and retinoic acid. These two factors function as triggers that alter the characteristics of UC-MSCs to those of neural lineage cells, and subsequent treatment with forskolin, bFGF, PDGF and neuregulin synergistically promote the differentiation of UC-MSCs into cells with Schwann cell characteristics. Overall, the use of these factors for inducing Schwann cells from MSCs mimics normal Schwann cell development and, thus, this system is generally efficient for inducing the differentiation of MSCs into Schwann cells.

Possible Use of Muse Cells, a Subpopulation of MSCs, for Efficient Generation of Schwann Cells

Recently, pluripotent stem cells, named multilineage-differentiating stress-enduring (Muse) cells, were discovered in MSCs such as BMSCs and ADSCs [Kuroda et al., 2010; Ogura et al., 2014]. Muse cells account for one to several percent of the total MSCs and, as is the case with MSCs, they are nontumorigenic and early realization of their application to regenerative medicine is highly anticipated.

Muse cells, which were initially identified as stress-tolerant cells, have remarkable characteristics. They express pluripotency genes, such as Oct3/4, Nanog, Sox2, Rex1, are able to differentiate into mesodermal-, ectodermal- and endodermal-lineage cells from a single cell and are self-renewable [Kuroda et al., 2013]. The markers of each cell lineage into which Muse cells are able to differentiate are: ectodermal (nestin, NeuroD, Musashi, neurofilament, microtubule-associated protein-2, tyrosinase, microphthalmia-associated transcription factor, gf100, tyrosinase-related protein 1 and dopachrome tautomerase), mesodermal (brachyury, Nkx2.5, smooth muscle actin, osteocalcin, FABP-4 and desmin), and endodermal lineages (GATA-6, α-fetoprotein, cytokeratin-7 and albumin) [Kuroda et al., 2010; Wakao et al., 2011; Tsuchi-

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yama et al., 2013; Ogura et al., 2014]. Expression of these markers is recognized under both spontaneous differentiation and cytokine induction systems.

While Muse cells tend to differentiate spontaneously more frequently into mesodermal-lineage cells (10~15%), their background lineage, than into ectodermal- or endodermal-lineage cells (3~4%), an induction system with a certain combination of cytokines directs their differentiation more efficiently [Kuroda et al., 2010]. For example, when they are treated with neurobasal medium supplemented with B-27, bFGF and brain-derived neurotrophic factor differentiate Muse cells into neuronal cells that are positive for MAP-2 and neurofilament [Wakao et al., 2011]. Therefore, Schwann cells are expected to be efficiently obtained from Muse cells, depending on the induction system.

Another outstanding character of Muse cells is that they have the ability to migrate to and integrate into the site of damage and then spontaneously differentiate into cells compatible with a wide spectrum of tissues that they target. Such ability was demonstrated by the infusion of green fluorescent protein-labeled naive human Muse cells (derived from BMSCs) into immunodeficient mouse (SCID mouse) models [Kuroda et al., 2010; Wakao et al., 2012]. Naive human Muse cells infused into the bloodstream of mouse models targeted damaged sites and differentiated into hepatocytes (Muse cells differentiated into cells that expressed human albumin), skeletal muscle cells (human dystrophin), neuronal cells (neurofilament) and keratinocytes (human cytokeratin 14), respectively [Kuroda et al., 2010; Wakao et al., 2012]. Since differentiation and repair are induced spontaneously by Muse cells themselves there is no need to control their differentiation prior to transplantation. This is a unique property that is not seen with other kinds of stem cells, such as embryonic stem and induced pluripotent stem cells. From this point of view, Muse cells collected from BMSCs and ADSCs are expected to repair spinal cord and PNS only by direct supply of the cells into the locus or the blood stream. However, all these possibilities need to be examined robustly in the future studies.

Future Perspective of MSC-Schwann Cells

Schwann cells are useful for clinical treatment of peripheral nerve injury, neuropathy, multiple sclerosis, spinal cord injury, and other neurotraumatic and neurologic diseases because they not only elicit and support axonal regeneration, but they are also able to form myelin, a crucial function for nervous system repair. MSCs offer great potential for cell transplantation therapy because of their easy accessibility and proliferative capacity. BMSCs are easily accessed by aspiration of the bone marrow and can be isolated from both healthy donors and patients, and expanded on a large scale. For example, 20–100 ml of bone marrow aspirate yields 1×10^7 BMSCs within several weeks, providing a sufficient number of cells for transplantation [Dezawa et al., 2004]. UC-MSCs and ADSCs are also major sources of MSCs. Mesenchymal tissue from the umbilical cord, so-called Wharton's jelly, contains an abundance of MSCs. The umbilical cord derives from postnatal tissue discarded after birth and thus collection of UC-MSCs is not an invasive procedure for donors or patients. A disadvantage of UC-MSCs, however, is that they are usually not applicable to autologous transplantation. ADSCs are another useful alternative source. They can be harvested by lipoaspiration, which is a safe and noninvasive procedure. Hundreds of millions of ADSCs can be isolated from only 1–2 liters of lipoaspirate [Heneidi et al., 2013].

MSCs derived from various sources can be induced to generate cells that acquire Schwann cell-like morphology in vitro and express Schwann cell-specific proteins. These cells can take up position immediately adjacent to axons and can release growth factors and cytokines that promote neurite outgrowth. These features are highly suggestive of the differentiation of functional Schwann cells. However, the formation of ensheathing myelin has only been demonstrated in a few studies. This needs to be investigated more thoroughly before functional myelin formation by MSC-derived Schwann-like cells can be considered to have a readily applicable therapeutic potential.

Since the first report of MSC induction into Schwann cells, many other groups have successfully generated MSC-Schwann cells from multiple sources. The basic method of differentiating Schwann cells from MSCs (β-mercaptoethanol → retinoic acid → bFGF + PDGF + forskolin + neuregulin) involves only cytokine treatment and no gene introduction. Thus, this system is expected to lower the hurdle for clinical application. Notably, autologous BMSC-Schwann cells were demonstrated to be effective and safe for PNS regeneration for up to 1 year when evaluated based on general conditions, tumor markers in blood analysis and ^{18}F-fluorodeoxyglucose-positron emission tomography [Wakao et al., 2010]. Furthermore, human sources, i.e. human BMSCs, UC-MSCs and ADSCs, are able to generate Schwann cells, which is an important point for clinical application. Therefore, MSC-Schwann cells are considered a strong viable alternative to authentic Schwann cells.

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RESEARCH PAPER

A nationwide survey of combined central and peripheral demyelination in Japan

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ABSTRACT

Objectives To clarify the clinical features of combined central and peripheral demyelination (CCPD) via a nationwide survey.

Methods The following characteristics were used to define CCPD: T2 high-signal intensity lesions in the brain, optic nerves or spinal cord on MRI, or abnormalities on visual-evoked potentials; conduction delay, conduction block, temporal dispersion or F-wave abnormalities suggesting demyelinating neuropathy based on nerve conduction studies; exclusion of secondary demyelination. We conducted a nationwide survey in 2012, sending questionnaires to 1332 adult and paediatric neurology institutions in Japan.

Results We collated 40 CCPD cases, including 29 women. Age at onset was 31.7±14.1 years (mean±SD). Sensory disturbance (94.9%), motor weakness (92.5%) and gait disturbance (79.5%) were common. Although cerebrospinal fluid protein levels were increased in 82.5%, oligoclonal IgG bands and elevated IgG indices were detected in 7.4% and 18.5% of cases, respectively. Fifteen of 21 patients (71.4%) had abnormal visual-evoked potentials. Antineurofascin 155 antibodies were positive in 5/11 (45.5%). Corticosteroids, intravenous immunoglobulins and plasmapheresis resulted in an 83.3%, 66.7% and 87.5% improvement, respectively, whereas interferon-β was effective in only 10% of cases. CCPD cases with simultaneous onset of central nervous system (CNS) and peripheral nervous system (PNS) involvement exhibited greater disability, but less recurrence and more frequent extensive cerebral and spinal cord MRI lesions compared to those with temporarily separated onset, whereas optic nerve involvement was more common in the latter.

Conclusions CCPD shows different characteristics from classical demyelinating diseases, and distinctive features exist between cases with simultaneous and temporarily separated onset of CNS and PNS involvement.

INTRODUCTION

Inflammatory demyelinating diseases are immune-mediated inflammatory disorders of the nervous system, which are divided into two categories: those affecting the central nervous system (CNS), such as acute disseminated encephalomyelitis and multiple sclerosis (MS) and those affecting the peripheral nervous system (PNS), including Guillain-Barré syndrome and chronic inflammatory demyelinating polyradiculoneuropathy (CIDP).

Demyelinating diseases usually affect either the CNS or PNS, possibly because the relevant autoimmune cells recognise only CNS or PNS antigens. However, it has occasionally been reported that patients with demyelination in the CNS or PNS also exhibit demyelination in the other nervous system. It was reported that 13 of 150 patients with MS had symptoms related to peripheral neuropathy and 4 had demyelinating polyneuropathy.[1] In addition, 5 of 100 patients with CIDP had symptomatic CNS involvement.[2] Demyelinating conditions affecting both the CNS and PNS are described using various diagnostic names, such as combined central and peripheral demyelination (CCPD), CIDP with CNS involvement and CIDP with multifocal CNS demyelination.[3] Although case reports or a small series of studies of such cases have been repeatedly found in the literature,[4–17] whether such conditions represent a distinct disease entity remains to be determined. Since large-scale epidemiological studies on this condition have never before been performed, we conducted a nationwide survey in Japan to uncover the demographic features of CCPD.

METHODS

Procedures

In this survey, CCPD was defined as fulfilling the following criteria:

1. CNS involvement criterion: T2 high-signal intensity lesions in the brain, optic nerves or spinal cord on MRI, or abnormalities on visual-evoked potentials (VEPs).
2. PNS involvement criterion: conduction delay, conduction block, temporal dispersion or F-wave abnormalities, suggesting peripheral demyelinating neuropathy according to nerve conduction studies (NCS). In the present study, it was mandatory that among median, ulnar and tibial nerves, at least two nerves had the aforementioned abnormal findings suggestive of demyelination.
3. Exclusion criterion: secondary demyelinating diseases or changes, such as infectious diseases (eg, human T lymphocyte trophic virus type 1-associated myelopathy, syphilis, neuroborreliosis, HIV infection or progressive multifocal leucoencephalopathy), pre-existing inflammatory diseases (eg, sarcoidosis, Behçet's disease, Sjögren's syndrome, vasculitis or other collagen diseases), mitochondrial disease, metabolic/toxic

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diseases (eg, vitamin deficiency, amyloidosis, chronic alcoholism, diabetes mellitus or subacute myelo-opticoneuropathy due to clioquinol intoxication, cervical spondylotic myelopathy, syringomyelia, spinocerebellar degeneration, multiple myeloma, other tumours, inherited diseases (eg, leucodystrophies), cerebrovascular disease and non-specific lesions on T2-weighted MRI (eg, leucoaraiosis). In our previous study on CCPD,[18] all seven CCPD cases fulfilled the EFNS/PNS criteria for CIDP and six cases met the McDonald criteria (2011) for MS.[19 20] Therefore, we did not exclude patients who eventually met either MS or CIDP criteria for the present survey.

Patients with CCPD who visited adult or paediatric neurologists between 2007 and 2011, and met the aforementioned diagnostic criteria were surveyed in 2012. The survey was conducted in two steps. First, a primary questionnaire sheet was sent to 1332 institutions in Japan, which included educational facilities accredited by the Japanese Society of Neurology, neurology departments with two or more board-certified neurologists, neurology departments in hospitals with more than 500 beds, paediatric departments in hospitals with any board-certified paediatric neurologist and departments of paediatrics in medical schools. A response was received from 671 institutions (50.3%), of which 41 institutions reported 57 cases. In the second step, a survey using a detailed questionnaire sheet about each patient was sent to the institutions that reported the CCPD cases. This questionnaire requested the age at onset, sex, history of preceding diseases, habitation area, mode of onset, clinical signs and symptoms, Hughes functional scale scores (grade 0: normal; grade 1: minimal symptoms and signs, able to run; grade 2: able to walk 5 m independently; grade 3: able to walk 5 m with the use of aids; grade 4: chairuser or bedbound; grade 5: requires assisted ventilation; grade 6: dead)[21] at the peak and in remission, laboratory findings, MRI findings of the brain and spinal cord, VEP and NCS findings, differential diagnosis, clinical course, treatment and outcomes. In this second survey, 54 of 57 cases (94.7%) were collated from 38 institutions (92.7%).

Among 54 cases collated, 14 cases were excluded for the following reasons: four cases did not meet CNS involvement criteria; four cases did not meet PNS involvement criteri/a; two cases lacked basic clinical data; two cases were experienced outside the term of this survey; and two cases were strongly suspected of having other diseases (cerebral vascular disease in one and leucodystrophy in another). In the present survey, CNS and PNS symptoms developed less than 2 months apart were regarded as simultaneous or sequential onset of both CNS and PNS involvement. The mode of onset was defined as acute (reaching a maximum intensity within 1 week), subacute (reaching a maximum intensity after 1 week to 1 month) or chronic (reaching a maximum intensity after 1 month).

Statistical analysis

Continuous variables were summarised by descriptive statistics, and categorical variables were summarised using counts of patients and percentages. For comparisons between two groups, qualitative variables were analysed using Fisher's exact test. Continuous variables that followed a parametric distribution were analysed with Student's t tests, whereas non-parametric variables were analysed with the Mann-Whitney U test.

Table 1 Demographic features of 40 patients with CCPD

Basic demographics	N=40
Sex ratio (male/female)	11:29
Age at onset (years, mean±SD)	31.7±14.1
Age at examination (years, mean±SD)	36.5±14.6
Follow-up period (months, mean±SD)*	93.0±91.8
Disease duration (months, mean±SD)*	137.9±124.8
Mode of onset	n/N (%)
Acute	6/31 (19.4)
Subacute	14/31 (45.2)
Chronic	11/31 (35.5)
Clinical course	n/N (%)
Monophasic	10/38 (26.3)
Relapse–remitting	20/38 (52.6)
Chronic progressive	8/38 (21.1)
Initial symptoms	n/N (%)
Related to CNS involvement	15/38 (39.5)
Related to PNS involvement	15/38 (39.5)
Simultaneous or sequential	8/38 (21.0)
Fulfilment of MS or CIDP criteria	n/N (%)
McDonald criteria for MS	27/40 (67.5)
EFNS/PNS definite criteria for CIDP	35/40 (87.5)
Symptoms and signs during the entire course	n/N (%)
Seizure†	3/40 (7.5)
Mental disturbance†	5/40 (12.5)
Visual disturbance†	19/40 (47.5)
Right	1/19 (5.3)
Left	8/19 (42.1)
Bilateral	10/19 (52.6)
Cranial nerve involvement (other than the optic nerves)	17/39 (43.6)
Motor weakness‡	37/40 (92.5)
Hemiplegia†	10/36 (27.8)
Paraplegia†	6/36 (16.7)
Weakness of 4 extremities§	24/36 (66.7)
Muscle atrophy§	11/40 (27.5)
Respiratory disturbance	3/40 (7.5)
Gait disturbance	31/39 (79.5)
Cerebellar ataxia†	10/38 (26.3)
Sensory disturbance	37/39 (94.9)
Half-body involvement†	5/37 (13.5)
Sensory level†	14/37 (37.8)
Glove and stocking type§	22/37 (59.4)
Other types	3/37 (8.1)
Deep tendon reflexes	
Hyporeflexia§	26/40 (65.0)
Normal	1/40 (2.5)
Hyper-reflexia†	9/40 (22.5)
Both hyporeflexia and hyper-reflexia	4/40 (10.0)
Pathological reflexes†	18/40 (45.0)
Sphincter disturbance†	18/38 (47.4)

*Two patients' data were missing.
†Symptoms derived from CNS involvement.
‡Detail of motor weakness in one patient was unknown.
§Symptoms derived from PNS involvement.
CCPD, combined central and peripheral demyelination; CIDP, chronic inflammatory demyelinating polyradiculoneuropathy; CNS, central nervous system; n, number of involved cases; N, number of cases collated; MS, multiple sclerosis; PNS, peripheral nervous system.

RESULTS

Baseline characteristics

The demographic features of 40 patients with CCPD are summarised in table 1. The mean age at onset was 31.7±14.1 years (range: 8–59 years), with disease duration of 137.9±124.8 months. The male to female ratio was 1:2.6 (11/29). The mode of onset was acute in 19.4%, subacute in 45.2% and chronic in 35.5%. Clinical courses were monophasic in 10 (26.3%), relapsing remitting in 20 (52.6%) and chronic progressive in 8 (21.1%)

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cases. Four patients had antecedent infections, of which three had respiratory infections and one had an alimentary infection. Only one patient developed CCPD after a vaccination (details of the vaccination are unknown). In the present survey, 67.5% (27/40) of the patients with CCPD met the McDonald criteria[20] for MS, while 87.5% (35/40) fulfilled the EFNS/PNS definite criteria for CIDP.[19]

Neurological symptoms and signs

The initial symptoms related to CNS involvement, such as visual disturbance, hemiplegia and hemibody sensory disturbance, were observed in 15 cases (39.5%), those related to PNS involvement, such as weakness and sensory disturbance of four extremities, in 15 cases (39.5%), and those related to both CNS and PNS involvement (simultaneous or sequential occurrence) in 8 cases (21%). The most common symptom/sign during the entire course was sensory disturbance (94.9%), the second most common symptom/sign was motor weakness (92.5%) and the third was gait disturbance (79.5%). Visual disturbance was observed in nearly half of the patients, with approximately 50% exhibiting bilateral involvement. Overall, cranial nerves were affected in 30/40 (75%) cases and optic nerves were the most commonly affected (19/30, 63.3%; see online supplementary table). Hyporeflexia and hyper-reflexia were seen in 65% and 22.5%, respectively, while four patients had both, depending on what was examined. Pathological reflexes were found in 45% and sphincter disturbance was present in 47.4%. About one-fourth of the patients showed muscle atrophy and cerebellar ataxia. Mental disturbance, seizure and respiratory disturbance were only occasionally observed.

Laboratory findings of peripheral blood and cerebrospinal fluid

Increased C reactive protein levels were found in only 10.5% of the cases and none of the patients had abnormal glycated haemoglobin levels (table 2). Few patients had common autoantibodies. Antiaquaporin 4 (AQP4) antibodies were not detected in any of the patients, whereas antineurofascin155 antibodies were found in 5/11 (45.5%). Epstein-Barr virus, herpes simplex virus, varicella zoster virus and mycoplasma were negative in all examined cases. Cerebrospinal fluid (CSF) protein levels were increased in 82.5% of the cases, while pleocytosis was present in 27.5%, indicating albuminocytological dissociation in 57.5%. The CSF oligoclonal IgG band positivity rate was only 7.4% and an elevated IgG index was found in 18.5% of the cases.

Table 2 Laboratory findings in 40 patients with CCPD

	n/N (%)
Blood	
High HbA1c level	0/37 (0)
CRP level >1.0 mg/dL	4/38 (10.5)
Hyperthyroidism	1/37 (2.7)
Hypothyroidism	3/37 (8.1)
Rheumatoid factor	1/31 (3.2)
ANA ≥1:160	1/31 (3.2)
Anti-SS-A Ab	0/35 (0)
Anti-SS-B Ab	0/35 (0)
MPO-ANCA	1/27 (3.7)
PR3-ANCA	0/25 (0)
Anti-AQP4 Ab	0/29 (0)
Antiganglioside Ab	2/24 (8.3)
Antineurofascin155 Ab	5/11 (45.5)
Monoclonal gammopathy	1/28 (3.6)
CSF	
Amounts of protein >40 mg/dL	33/40 (82.5)
Cell counts >5/μL	11/40 (27.5)
Albuminocytological dissociation	23/40 (57.5)
OB	2/27 (7.4)
Increased IgG index level	5/27 (18.5)

Ab, antibodies; ANA, antinuclear antibody; AQP4, aquaporin 4; CCPD, combined central and peripheral demyelination; CRP, C reactive protein; CSF, cerebrospinal fluid; HbA1c, glycated haemoglobin; MPO-ANCA, myeloperoxidase-antineutrophil cytoplasmic antibody; N, number of cases collated; N, number of involved cases; OB, oligoclonal IgG bands; PR3-ANCA, proteinase-3-antineutrophil cytoplasmic antibody.

Neuroimaging, VEP and NCS findings

Following MRI examination, cerebral, cerebellar, brainstem and optic nerve lesions were detected in 75%, 15%, 32.5% and 17.5%, respectively (table 3). Among cases with cerebral lesions, 36.7% had nine or more lesions. Large lesions (>3 cm in diameter) were observed in 25% and gadolinium (Gd)-enhanced lesions were found in only 17.5%. Spinal cord lesions were found in 30/40 (75%) and the lesions in 11 cases were Gd-enhanced. Longitudinally extensive spinal cord lesions (LESCLs), extending three or more vertebral segments, were present in 3/40 (7.5%). VEPs were abnormal in 15/21 patients (71.4%) and bilaterally observed in 53.3% of these. Based on neurological, MRI and VEP findings, the involvement of multiple affected CNS sites (either two or three sites among the brain, optic nerves and spinal cord) was found in 70% of

Table 3 MRI and VEP findings in 40 patients with CCPD

	n/N (%)
MRI	
Cerebral lesions	30/40 (75.0)
≤3	6/30 (20.0)
4–8	13/30 (43.3)
≥9	11/30 (36.7)
Gd-enhancement	7/40 (17.5)
Lesions larger than 3 cm	10/40 (25.0)
Cerebellar lesions	6/40 (15.0)
Gd-enhancement	2/40 (5.0)
Brainstem lesions	13/40 (32.5)
Gd-enhancement	3/40 (7.5)
Optic nerve lesions	7/40 (17.5)
Gd-enhancement	1/40 (2.5)
Spinal cord lesions	30/40 (75.0)
LESCLs	3/40 (7.5)
Gd-enhancement	11/40 (27.5)
VEPs	
Abnormal findings	15/21 (71.4)
Right	2/15 (13.3)
Left	5/15 (33.3)
Bilateral	8/15 (53.3)
Affected CNS sites	
Brain only	4/40 (10.0)
Optic nerves only	1/40(2.5)
Spinal cord only	7/40 (17.5)
Brain+optic nerves	5/40 (12.5)
Brain+spinal cord	13/40 (32.5)
Optic nerves+spinal cord	2/40 (5.0)
Brain+optic nerves+spinal cord	8/40 (20.0)

CCPD, combined central and peripheral demyelination; CNS, central nervous system; Gd, gadolinium; LESCLs, longitudinally extensive spinal cord lesions; N, number of cases collated; N, number of involved cases; VEPs, visual-evoked potentials.

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Table 4 Abnormal findings of NCS in 40 patients with CCPD

	Total†	Median‡	Ulnar‡	Tibial‡	Sural‡
Motor nerve					
Decreased MCV	31/40 (77.5)	55/69 (79.7)	47/66 (71.2)	46/63 (73.0)	
Prolonged distal latency	21/40 (52.5)	31/67 (46.3)	28/62 (45.2)	22/59 (37.3)	
Decreased or absent CMAP	22/40 (55.0)	19/70 (27.1)	26/69 (37.7)	44/70 (62.9)	
Conduction block	11/40 (27.5)	20/64 (31.3)	22/61 (36.1)	20/59 (33.9)	
Temporal dispersion	16/40 (40.0)	23/67 (34.3)	27/64 (42.2)	23/58 (39.7)	
Prolonged F-wave latency	28/40 (70.0)	38/54 (70.4)	29/45 (64.4)	34/41 (82.9)	
Decreased F-wave occurrence	19/40 (47.5)	28/58 (48.3)	24/50 (48.0)	21/50 (42.0)	
Sensory nerve					
Decreased SCV	17/40 (42.5)	20/53 (37.7)	30/49 (61.2)		17/38 (44.7)
Decreased or absent SNAP	35/40 (87.5)	41/66 (62.1)	50/68 (73.5)		43/60 (71.7)

†Patients with indicated abnormalities in any one of the three nerves were regarded as abnormal (numbers of abnormal patients/total numbers of patients examined).
‡Numbers of abnormal nerves/total numbers of nerves examined.
CCPD, combined central and peripheral demyelination; CMAP, compound muscle action potential; MCV, motor nerve conduction velocity; NCS, nerve conduction study; SCV, sensory nerve conduction velocity; SNAP, sensory nerve action potential.

patients with CCPD, while isolated involvement of the brain, optic nerve lesions or spinal cord was present in 10%, 2.5% and 17.5%, respectively. Devic type (optic-spinal) involvement was observed in only 5%. In motor NCS, decreased motor nerve conduction velocity and prolonged F-wave latency were the most common findings, and were observed in 77.5% and 70% of patients with CCPD, respectively (table 4). Abnormal compound muscle action potential amplitude, prolonged distal latency and decreased F-wave occurrence were detected in approximately half of the patients. Conduction block and temporal dispersion were detected in 27.5% and 40%, respectively. In sensory NCS, decreased or absent sensory nerve action potential was recognised in as much as 87.5%, while decreased sensory nerve conduction velocity was present in 42.5%.

Treatment and prognosis

Patients with CCPD were most commonly treated with either intravenous or oral corticosteroids, followed by intravenous immunoglobulins, resulting in 83.3%, 75% and 66.7% improvement, respectively (table 5). Plasmapheresis was performed in only eight patients, of whom seven (87.5%) improved. By contrast, interferon-β (IFN-β) was effective in only one patient and the disease was actually exacerbated in three patients. At the illness peak, 16/40 (40%) patients with CCPD had severe disability, with a Hughes functional scale score of 4 or more, and three required artificial ventilation (figure 1). However, after treatment, 26 of 40 (65%) patients had no or only mild disabilities (≤1 Hughes functional scale score).

Table 5 Treatment response in 40 patients with CCPD

Treatment	Efficacy n/N (%)
Corticosteroid pulse therapy*	30/36 (83.3)
Oral corticosteroids	21/28 (75.0)
IVIg	18/27 (66.7)
Plasmapheresis	7/8 (87.5)
IFN-β	1/10 (10.0)

*500 mg/day for three consecutive days were administered to two patients, while 1000 mg/day for three consecutive days were administered to the remaining patients.
CCPD, combined central and peripheral demyelination; IFN-β, interferon β; IVIg, intravenous immunoglobulin; N, number of cases collated; n, number of efficacious cases.

Comparison of clinical features between patients with CCPD with simultaneous or temporarily separated onset of CNS and PNS involvement

We classified the collated patients into two subgroups according to the pattern of onset: simultaneous or sequential involvement of both CNS and PNS at onset (simultaneous onset group), or temporarily separated onset of CNS and PNS involvement (temporarily separated onset group). Follow-up period and disease duration were significantly shorter in the simultaneous onset group than in the temporarily separated onset group (44.6 ±45.0 months vs 112.0±97.7 months, p=0.0316 and 56.9 ±58.2 vs 169.3±128.5 months, p=0.0055, respectively; table 6). In the temporarily separated onset group, patients who had already been diagnosed as MS when PNS demyelination developed were seen in 9/15 (60%), while those who had already been diagnosed as CIDP when CNS demyelination developed were seen in 7/15 (46.7%) cases. The Hughes functional scale scores at the peak of illness were significantly greater in the simultaneous onset group than the temporarily separated onset

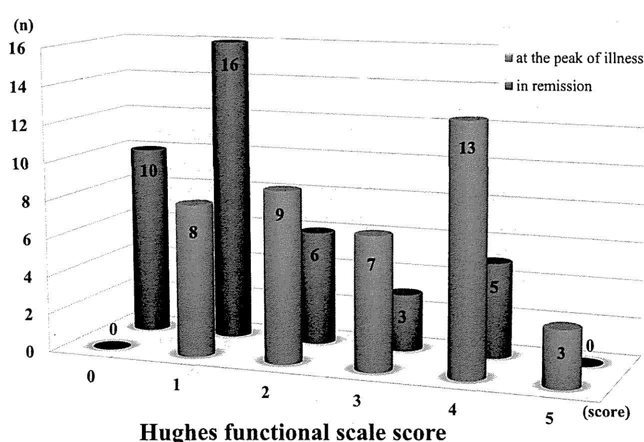

Figure 1 Hughes functional scale scores at the peak of illness and in remission. Forty patients with combined central and peripheral demyelination were evaluated by the Hughes functional scale score at the peak of illness and in remission. No one died because of the disease. The post-treatment scores became significantly less than the pretreatment scores (2.85±1.29 to 1.43±1.30, p<0.0001). All three patients with grade 5 at the peak of illness belonged to the simultaneous onset group.

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Table 6 Comparison of clinical features between patients with CCPD with simultaneous or temporarily separated onset of CNS and PNS involvement*

	Temporarily separated onset group	Simultaneous onset group	p Value†
Demographics	N=30	N=8	
Sex ratio (male/female)	7:23 (1:3.3)	2:6 (1:3)	NS
Age at onset (years, mean±SD)	29.4±13.2	35.0±14.9	NS
Age at examination (years, mean±SD)	35.5±14.8	36.0±14.1	NS
Follow-up period (months, mean±SD)‡	112.0±97.7	44.6±45.0	0.0316
Disease duration (months, mean±SD)‡	169.3±128.5	56.9±58.2	0.0055
Mode of onset	n/N (%)	n/N (%)	
Acute	4/22 (18.2)	2/8 (25.0)	NS
Subacute	9/22 (40.9)	4/8 (50.0)	NS
Chronic	9/22 (40.9)	2/8 (25.0)	NS
Clinical course	n/N (%)	n/N (%)	
Monophasic	3/29 (10.3)	6/8 (75.0)	0.0008
Relapsing–remitting	19/29 (65.5)	1/8 (12.5)	0.0140
Chronic progressive	7/29 (24.1)	1/8 (12.5)	NS
Fulfilment of MS or CIDP criteria	n/N (%)	n/N (%)	
McDonald criteria for MS	22/30 (73.3)	4/8 (50.0)	NS
EFNS/PNS definite criteria for CIDP	26/30 (86.7)	7/8 (87.5)	NS
The number of patients who had already been diagnosed as MS when PNS demyelination developed	9/15 (60.0)		
The number of patients who had already been diagnosed as CIDP when CNS demyelination developed	7/15 (46.7)		
Hughes functional scale score	N=30	N=8	
At the peak of illness	2.73±1.14	3.75±1.39	0.0457
In remission	1.43±1.28	1.50±1.60	NS
Score changes after treatment	1.30±0.99	2.25±1.16	0.0427
Symptoms and signs	n/N (%)	n/N (%)	
Visual disturbance	19/30 (63.3)	0/8 (0.0)	0.0015
Cranial nerve involvement (other than optic nerves)	12/29 (41.4)	5/8 (62.5)	NS
Motor weakness	29/30 (96.7)	7/8 (87.5)	NS
Muscle atrophy	9/30 (30.0)	2/8 (25.0)	NS
Respiratory disturbance	0/30 (0.0)	3/8 (37.5)	0.0066
Gait disturbance	22/29 (75.9)	7/8 (87.5)	NS
Cerebellar ataxia	8/30 (26.7)	2/6 (33.3)	NS
Sensory disturbance	30/30 (100.0)	5/7 (71.4)	0.0315
Pathological reflexes	13/30 (43.3)	5/8 (62.5)	NS
Sphincter disturbance	14/29 (48.3)	3/7 (42.9)	NS
Blood	n/N (%)	n/N (%)	
Antineurofascin 155 Ab	3/8 (37.5)	2/3 (66.7)	NS
CSF	N=30	N=8	
Amounts of protein	85.3±64.9	126.5±88.3	NS
Cell counts	4.61±6.06	26.0±52.3	NS
	n/N (%)	n/N (%)	
Amounts of protein >40 mg/dL	24/30 (80.0)	7/8 (87.5)	NS
Cell counts >5/μL	7/30 (23.3)	3/8 (37.5)	NS
OB	2/21 (9.5)	0/5 (0.0)	NS

Continued

Table 6 Continued

	Temporarily separated onset group	Simultaneous onset group	p Value†
Increased IgG index level	4/20 (20.0)	1/6 (16.7)	NS
MRI	n/N (%)	n/N (%)	
Brain lesions	23/30 (76.7)	8/8 (100.0)	NS
Cerebral lesions	21/30 (70.0)	8/8 (100.0)	NS
Lesions more than 3 cm	5/30 (16.7)	5/8 (62.5)	0.0186
Cerebellar lesions	6/30 (20.0)	0/8 (0.0)	NS
Brainstem lesions	10/30 (33.3)	2/8 (25.0)	NS
Optic nerve lesions	6/30 (20.0)	1/8 (12.5)	NS
Spinal cord lesions	24/30 (80.0)	4/8 (50.0)	NS
LESCLs	0/30 (0.0)	3/8 (37.5)	0.0066
VEPs	n/N (%)	n/N (%)	
Abnormal VEP findings	14/17 (82.4)	1/4 (25.0)	0.0526§
Affected CNS sites	n/N (%)	n/N (%)	
Brain only	1/30 (3.3)	3/8 (37.5)	0.0237
Optic nerves only	1/30 (3.3)	0/8 (0.0)	NS
Spinal cord only	6/30 (20.0)	0/8 (0.0)	NS
Brain+optic nerves	4/30 (13.3)	1/8 (12.5)	NS
Brain+spinal cord	9/30 (30.0)	3/8 (37.5)	NS
Optic nerves+spinal cord	2/30 (6.7)	0/8 (0.0)	NS
Brain+optic nerves+spinal cord	7/30 (23.3)	1/8 (12.5)	NS
Treatment efficacy	n/N (%)	n/N (%)	
Corticosteroid pulse therapy	25/27 (92.6)	6/8 (75.0)	NS
Oral corticosteroids	17/20 (85.0)	4/6 (66.7)	NS
IVIg	13/20 (65.0)	4/5 (80.0)	NS
Plasmapheresis	5/6 (83.3)	2/2 (100.0)	NS

*Two patients were excluded because their patterns of onset were undetermined.
†A p value<0.05 is regarded as significant. Qualitative variables were analysed by Fisher exact test. Continuous variables that follow a parametric distribution were analysed by Student's t test, while non-parametric variables were analysed by Mann-Whitney U test.
‡Two patients' data in the temporarily separated onset group were missing.
§Indicates a trend (ie, p<0.1).
Ab, antibodies; CCPD, combined central and peripheral demyelination; CIDP, chronic inflammatory demyelinating polyradiculoneuropathy; CNS, central nervous system; CSF, cerebrospinal fluid; IFN-β, interferon β; IVIg, intravenous immunoglobulin; LESCLs, longitudinally extensive spinal cord lesions; MS, multiple sclerosis; N, number of cases collated; n, number of involved cases; NS, not significant; OB, oligoclonal IgG bands; PNS, peripheral nervous system; VEPs, visual-evoked potentials.

group (2.73±1.14 vs 3.75±1.39, p=0.0457). The monophasic course was more frequently observed in the simultaneous onset group than the temporarily separated onset group (75% vs 10.3%, p=0.0008), whereas the relapsing–remitting course was more common in the temporarily separated onset group than the simultaneous onset group (65.5% vs 12.5%, p=0.0140). Visual disturbance and sensory disturbance were more commonly present in the temporarily separated onset group than the simultaneous onset group (63.3% vs 0%, p=0.0015 and 100% vs 71.4%, p=0.0315, respectively), while respiratory disturbance occurred more often in the simultaneous onset group than in the temporarily separated onset group (37.5% vs 0%, p=0.0066). On MRI, cerebral lesions >3 cm and LESCLs were more frequently found in the simultaneous onset group than in the temporarily separated onset group (62.5% vs 16.7%, p=0 0.0186, and 37.5% vs 0%, p=0.0066, respectively). For the CNS affected sites, there were significantly more patients with PNS involvement and isolated brain involvement in the simultaneous onset group than in the temporarily separated onset

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group (37.5% vs 3.3%, p=0.0237). By contrast, no patients in the simultaneous onset group had PNS involvement and isolated spinal cord involvement, while six patients in the temporarily separated group showed PNS and isolated spinal cord involvement. Abnormal VEPs tended to be more frequently detected in the temporarily separated onset group than in the simultaneous onset group (82.4% vs 25%, p=0.0526). The Hughes functional scale scores were significantly lower following immunotherapies compared with pretreatment scores in the temporarily separated onset group and the simultaneous onset group (2.73 ±1.14 to 1.43±1.28, p=0.0002, and 3.75±1.39 to 1.50±1.60, p=0.0203, respectively). However, the improvement in these scores was more remarkable in the simultaneous onset group than in the temporarily separated onset group (2.25±1.16 vs 1.30±0.99, p=0.0427; figure 2). Even when we excluded the patient with a history of vaccination, we obtained essentially the same results, although the difference in the Hughes grade scores at the peak, and the score changes after treatment between the temporarily separated onset group and the simultaneous onset group, were no longer statistically significant because of the smaller sample size (data not shown).

DISCUSSION

CCPD is an extremely rare and devastating disease. We identified 40 patients throughout Japan during the study period. The numbers of registered MS and patients with CIDP in Japan in 2011 were 16 140 and 2986, respectively.[22] Even taking into consideration the response rates (50.3% in the first survey and 94.7% in the second), patients with CCPD were a very minor population (84 at most) among those with idiopathic demyelinating disorders (likely less than 0.52% of MS and 2.8% of patients with CIDP in Japan). The present nationwide survey is valuable for determining the characteristic features of CCPD. However, the study had some limitations. Many neurologists answered the questionnaires before the CCPD diagnostic criteria were established. In addition, because there are no specific biomarkers for either MS or CIDP, we could not differentiate these conditions from CCPD; instead, the number of patients who eventually met either the established MS or CIDP criteria was indicated. Nevertheless, the present study analysing the largest number of patients with CCPD defined by the same criteria is significant.

According to results from this study, CCPD was found in a preponderance of females and young adults. However, the age of onset ranged from 8 to 59 years, suggesting CCPD occurrence in a wide age range, except for elderly people. Thus, the ages of onset for CCPD overlap with those for MS and CIDP. Subacute or chronic onset was observed more often than acute onset, while a relapsing remitting or chronic progressive course was more common than a monophasic course. This suggested that a persisting inflammation affecting both the CNS and PNS was the main form of the disease. Indeed, most patients with CCPD reported in the literature to date show a relapsing remitting or chronic progressive course.[4–6 8–11 13–16] Initial symptoms that related to either CNS or PNS involvement were equally observed. CCPD had very high frequencies of motor weakness (>90%), as well as sensory disturbance with various distributions. Cranial nerve involvement that included optic nerves was also commonly seen in CCPD (75%).

The presence of widespread peripheral demyelination, as revealed by NCS and high frequency of albuminocytological dissociation, is compatible with CIDP. The abundant discrete CNS lesions which include the optic nerves and spinal cord on MRI are consistent with MS. However, several features distinct from

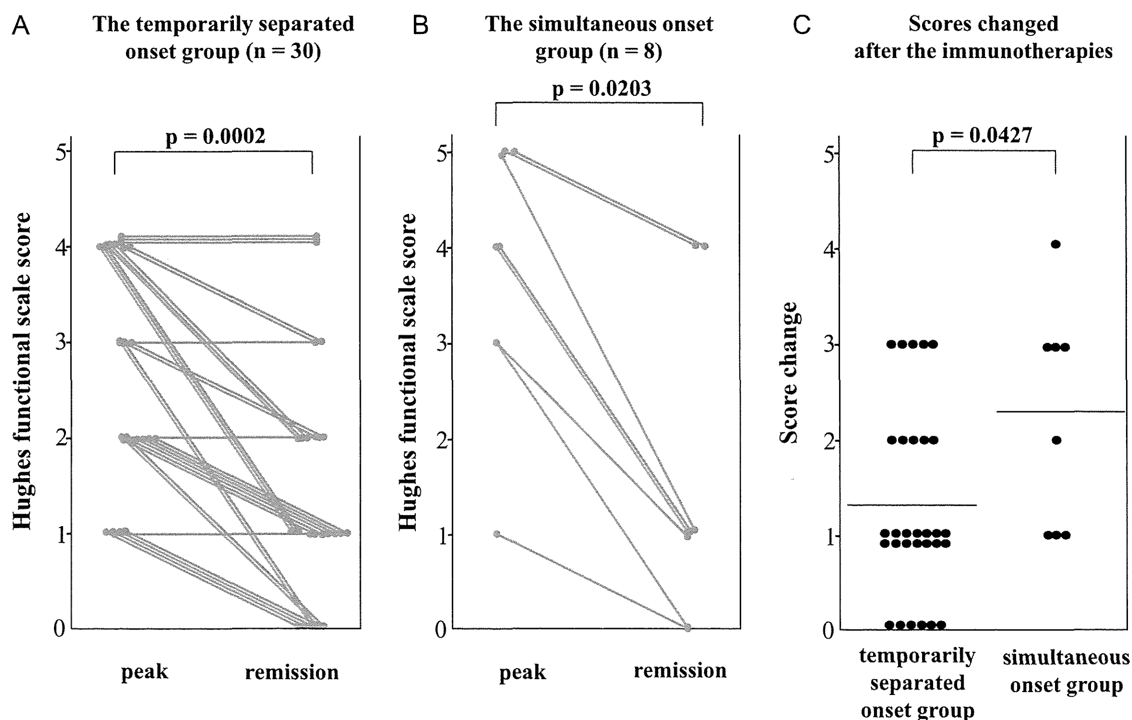

Figure 2 Comparison of treatment response in patients with combined central and peripheral demyelination with temporarily separated onset and those with simultaneous onset of central nervous system; and peripheral nervous system involvement. CCPD Hughes functional scale scores were significantly lower after immunotherapies compared with pretreatment scores in the temporarily separated onset group and simultaneous onset group (2.73±1.14 to 1.43±1.28, p=0.0002 and 3.75±1.39 to 1.50±1.60, p=0.0203, respectively). By contrast, score changes were more prominent in the simultaneous onset group than in the temporarily separated onset group (2.25±1.16 vs 1.30±0.99, p=0.0427). n=30 in the temporarily separated onset group and n=8 in the simultaneous onset group.

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MS were observed in the present study, including a low frequency of CSF IgG abnormalities and poor response to IFN-β. Zéphir *et al*[16] also reported an absence of CSF oligoclonal IgG bands in five cases with CCPD. Collectively, these findings suggest that at least some mechanisms are distinct from MS function in CCPD.

Most patients with CCPD responded well to immunotherapies, regardless of acute or chronic onset, suggesting a contributory immune/inflammatory mechanism. Although we found only one paper reporting on the efficacy of the combined use of intravenous immunoglobulin and plasma exchange in a case of CCPD,[12] the present study disclosed for the first time a high efficacy for plasma exchange in CCPD, which may suggest humoral immunity involvement. A female preponderance in CCPD is also consistent with the nature of autoimmune diseases, although common systemic autoantibodies and anti-ganglioside antibodies were infrequent, as in previous reports.[8–12 16 23] The unresponsiveness or even disease exacerbation following IFN-β therapy found in our study was consistent with previous reports of CIDP development after IFN-β introduction in patients with MS.[24 25] Such a phenomenon may also support an autoantibody-mediated mechanism because type I IFNs potently stimulate the production of all subclasses of IgG antibodies.[26] These findings suggest the involvement of specific autoantibodies reactive to antigens that are commonly present in CNS and PNS tissues in CCPD. Additional large-scale studies will be needed to clarify the relevant antigens in this condition.

There were several distinctive features between cases of simultaneous and temporarily separated onset of CCPD. A relapsing remitting course was observed more often in the latter than in the former, whereas a monophasic course was observed more often in the simultaneous onset. This difference may be because of the classification criteria as well as the shorter observation period of the simultaneous onset group. However, Adamovic *et al*[17] reported that among 13 paediatric patients with acute simultaneous inflammatory demyelination of both the CNS and PNS, recurrence was seen only in 2 (15.4%) cases. Accordingly, as in our series, the simultaneous onset cases were followed-up nearly 4 years on average. Therefore, simultaneous onset CCPD may be less likely to recur. In addition, the difference in clinical and laboratory manifestations cannot be explained solely by the difference in observation times. For example, visual disturbance and VEP abnormalities were observed more frequently in the temporarily separated onset group than in the simultaneous onset group, in which none of the cases showed apparent visual impairment. By contrast, frequencies of other cranial nerve involvements did not differ between the simultaneous onset and temporarily separated onset groups. Thus, frequent optic nerve involvement appears to be one characteristic feature of CCPD with temporarily separated onset. This suggestion is consistent with previous case reports examining relapsing CIDP with optic nerve lesions,[27–29] as well as the relatively high frequency of VEP abnormalities in relapsing or progressive patients with CIDP (8/17, 47%).[23] Therefore, this may be a useful laboratory test for suspected CCPD cases, especially those with relapsing CIDP as a presenting feature. By contrast, the simultaneous onset group had a significantly higher frequency of patients with PNS involvement and isolated brain involvement than the temporarily separated onset group, and no patients in the simultaneous onset group had PNS involvement and isolated spinal cord involvement, whereas 20% of the temporarily separated group patients did. Collectively, such differences in the CNS sites of involvement further indicate that distinct mechanisms are operating in these two subgroups.

It is interesting to note that LESCLs were exclusively found in the simultaneous onset group and extensive cerebral lesions were also more common in the simultaneous onset group than in the temporarily separated onset group. Since no AQP4 antibodies were detected in any CCPD cases examined, LESCLs are likely produced by a mechanism distinct from that in neuromyelitis optica (NMO). Indeed, Devic type (optic–spinal) involvement was seen only in the temporarily separated group but not in the simultaneous onset group, further suggesting that LESCLs in the simultaneous onset group are produced by mechanisms distinct from those in NMO. Although the mechanisms for such extensive lesions remain unknown, it is important to raise CCPD as a differential diagnosis for LESCLs and extensive brain lesions.

In the present series, compared with the temporarily separate onset cases, the simultaneous onset cases exhibited more severe disabilities at the peak of illness, such as higher frequencies of respiratory disturbance and greater Hughes functional scale scores, which were likely a reflection of the high frequency of extensive brain and spinal cord MRI lesions. These findings were consistent with those of Adamovic *et al*,[17] who showed that among 13 paediatric patients with acute simultaneous inflammatory demyelination of the CNS and PNS, 6 were bedbound or wheelchair users and one remained on mechanical ventilation at discharge. In our series, however, the simultaneous onset group showed improvements similar to or better than the temporarily separated onset group after immunotherapy, suggesting a high efficacy of immunotherapy for simultaneous onset CCPD, despite severe manifestations. Further studies and characterisation of simultaneous onset and temporarily separated onset CCPD cases may support the existence of two CCPD subtypes and help to shed light on the distinct mechanisms between the two subtypes.

In conclusion, CCPD exhibits distinctive features from those of the classical demyelinating diseases and, therefore, may be a distinct disease, but it is not a simple coexistence of MS and CIDP. Simultaneous onset CCPD is characterised by severe disability with a relatively high frequency of respiratory disturbance, as well as extensive brain and spinal cord lesions observed in MRI scans. By contrast, temporarily separated onset CCPD features a high frequency of optic nerve involvement. Although CCPD is extremely rare, awareness of this condition is important because responses to disease-modifying drugs, such as IFN-β, for patients with CCPD are different from those in patients with MS, and appropriate immunotherapies may well produce satisfactory outcomes with minimal disabilities.

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Contributors HO, DM, TY and JK conceived the study, supervised the analyses and wrote the paper. RY, NK, TM, MH and HM participated in procedure development and collated the data.

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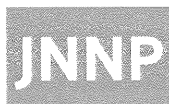

A nationwide survey of combined central and peripheral demyelination in Japan

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RESEARCH ARTICLE

Peripheral Blood T Cell Dynamics Predict Relapse in Multiple Sclerosis Patients on Fingolimod

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Abstract

Background

Fingolimod efficiently reduces multiple sclerosis (MS) relapse by inhibiting lymphocyte egress from lymph nodes through down-modulation of sphingosine 1-phosphate (S1P) receptors. We aimed to clarify the alterations in peripheral blood T cell subsets associated with MS relapse on fingolimod.

Methods/Principal Findings

Blood samples successively collected from 23 relapsing-remitting MS patients before and during fingolimod therapy (0.5 mg/day) for 12 months and 18 healthy controls (HCs) were analysed for T cell subsets by flow cytometry. In MS patients, the percentages of central memory T ($CCR7^{+}CD45RO^{+}$) cells (TCM) and naïve T ($CCR7^{+}CD45RO^{-}$) cells decreased significantly, while those of effector memory T ($CCR7^{-}CD45RA^{-}$) and suppressor precursor T ($CD28^{-}$) cells increased in both $CD4^{+}$T and $CD8^{+}$T cells from 2 weeks to 12 months during fingolimod therapy. The percentages of regulatory T ($CD4^{+}CD25^{high}CD127^{low}$) cells in $CD4^{+}$T cells and $CCR7^{-}CD45RA^{+}$T cells in $CD8^{+}$T cells also increased significantly. Eight relapsed patients demonstrated greater percentages of $CD4^{+}$TCM than 15 non-relapsed patients at 3 and 6 months (p=0.0051 and p=0.0088, respectively). The IL17-, IL9-, and IL4-producing $CD4^{+}$T cell percentages were significantly higher at pre-treatment in MS patients compared with HCs (p<0.01 for all), while the IL17-producing $CD4^{+}$T cell percentages tended to show a transient increase at 2 weeks of fingolimod therapy (p^{corr}=0.0834).

Conclusions

The $CD4^{+}$TCM percentages at 2 weeks to 12 months during fingolimod therapy are related to relapse.

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Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system and is assumed to be an autoimmune disease targeting myelin [1]. Fingolimod efficiently reduces MS relapse by inhibiting lymphocyte egress from lymph nodes through down-modulation of sphingosine 1-phosphate (S1P) receptors [2,3], which is consistent with findings in an animal model of MS that fingolimod successfully reduced the infiltration of myelin antigen-specific $CD4^{+}$T cells into inflammatory sites in experimental autoimmune encephalomyelitis [4]. Fingolimod traps $CD45RO^{+}$ central memory T cells (TCM) and naïve T cells expressing homing receptors for lymph nodes, CCR7 and CD62L, within lymph nodes [5]. The drug is supposed to exert its effects by inhibiting recirculation of TCM, which include autoreactive T cells [6]. Although fingolimod was also found to be beneficial in a controlled trial for human renal transplantation [7], its effects have not been examined for any other autoimmune diseases in large-scale clinical trials.

MS is now assumed to be a T helper 1 (Th1)/Th17-mediated autoimmune disease [1]. Some authors reported a decrease in IL17-positive cells in MS patients on fingolimod 1.25 mg once daily at the long-term therapy stage, but not on IFNβ [8], while others reported an increase in IL17-producing cells in a considerable fraction of MS patients on fingolimod 0.5 mg once daily at the short-term treatment stage [9]. In addition, effector memory T cells (TEM) without CCR7, which reside in non-lymphoid organs and mainly comprise IFNγ-producing Th1 cells protecting against infection, were found to show a relative increase during fingolimod therapy [7], while other authors reported TEM as the principal IL17-producing T cells [10]. Thus, the short-term and long-term effects of fingolimod on Th17 cells at an ordinary dosage (0.5 mg/day) remain to be elucidated. Therefore, we aimed to clarify the alterations in peripheral blood T cell subsets during short-term and long-term treatment periods, which are associated with therapeutic efficacy or treatment failure including suboptimal responses during fingolimod therapy.

Materials and Methods

Patients and control subjects

Venous blood samples were obtained from 18 healthy controls (HCs) (12 females and 6 males; mean age ± SD, 41.2±12.2 years) and 23 relapsing-remitting MS patients (15 females and 8 males; mean age ± SD, 42.3±12.5 years) who were started on fingolimod therapy between 2012 and 2014 at Kyushu University Hospital after informed consent was obtained (Table 1). In the latter, blood was withdrawn before and at 2 weeks and 1, 2, 3, 6, and 12 months after initiation of fingolimod 0.5 mg once daily. MS was diagnosed based on the 2005 revised McDonald criteria for MS [11] and all patients were seronegative for anti-aquaporin-4 (AQP4) antibodies [12,13]. Ten patients had neither immunomodulatory drugs nor corticosteroids within 3 months before the initiation of fingolimod, two had IFNβ-1b until 10 days and 1 day before fingolimod initiation, respectively, one had IFNβ-1a until 14 days before, and nine had prednisolone until 1 day before. All MS patients underwent a neurological examination at our outpatient clinic at 2–4-week intervals and were evaluated by the Expanded Disability Status Scale of Kurtzke (EDSS) [14] with calculation of the Progression Index [15], and observed for the emergence of clinical relapse. Clinical relapse was defined as the appearance of new symptoms or return of old symptoms for a period of 24 hours or more in the absence of a change in the core body temperature or infection [16]. Brain and spinal cord MRI was performed at 6 and 12 months after initiation of fingolimod to determine the presence of new/enlarging T2 lesions and gadolinium-enhanced T1 lesions (MRI relapse) [16]. All MRI studies were

Table 1. Demographic features of the subjects.

	HCs	MS PT	MS during fingolimod treatment for 12 months Relapsed patient	Non-relapsed patient
Sex (male: female)	6: 12	8: 15	3: 5	5: 10
Age at examination (mean ± SD, years)	41.2 ± 12.2	42.3 ± 12.5	35.6 ± 13.3	40.0 ± 11.9
Age at Onset (mean ± SD, years)	NA	28.9 ± 12.4	28.1 ± 12.4	28.7 ± 12.8
Disease duration (mean±SD, years)	NA	11.2 ± 9.7	7.3 ± 8.1	13.2 ± 10.1
Annualised relapse rate (mean ± SD, /years)	NA	3.3 ± 3.0	2.9 ± 3.0	3.5 ± 3.1
EDSS scores before fingolimod treatment (mean ± SD)	NA	2.8 ± 2.1	2.5 ± 2.3	3.2 ± 2.1
Progression Index (mean ± SD)	NA	0.6 ± 1.0	0.9 ± 1.6	0.4 ± 0.4

Abbreviations: HCs = healthy controls; MS PT = multiple sclerosis at pre-treatment; EDSS = Expanded Disability Status Scale of Kurtzke.

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performed using a 1.5 T unit (Magnetom Vision and Symphony; Siemens Medical Systems, Erlangen, Germany) as previously described [17]. This study was approved by the Kyushu University Hospital Ethics Committee. All individuals involved in this study signed a written informed consent form (Approval Number: 575–03).

Antibodies and flow cytometry

T cells were analysed in whole blood specimens using the following antibodies from Miltenyi Biotec (Auburn, CA): anti-human CD3-VioBlue (BW264/56, MACS), CD4-APC (M-T466, MACS), CD8-APC (BW135/80, MACS), CD45RO-FITC (UCHL1, MACS), CD45RA-APC-Vio770 (T6D11, MACS), CD127-FITC (MB15-18C9, MACS), CD25-PE (4E3, MACS), CCR7-PE (FR11-11E8, MACS), CD8-PE (BW135/80, MACS), CD4-FITC (M-T466, MACS), and CD28-APC (15E8, MACS). The following isotype controls from Miltenyi Biotec were also used: anti-mouse IgG1-PE (IS5-21F5, MACS), IgG1-APC (IS5-21F5, MACS), IgG1-FITC (IS5-21F5, MACS), IgG2a-APC (S43.10, MACS), IgG2a-VioBlue (S43.10, MACS), IgG2a-PE (S43.10, MACS), IgG2a-FITC (S43.10, MACS), IgG2b-APC-Vio770 (IS6-11E5.11, MACS), IgG2b-PE (IS6-11E5.11, MACS), and IgG2b-FITC (IS6-11E5.11, MACS). Specific cytokine-producing cells and chemokine receptor-positive cells were analysed using the following antibodies: anti-human CD4-APC (M-T466, MACS; Miltenyi Biotec), CD8-VioBlue (BW135/80, MACS; Miltenyi Biotec), IFNγ-FITC (25723.11; BD Biosciences, San Jose, CA), IL17-PE (BL168; Biolegend, San Diego, CA), IL4-FITC (MP4-25D2; Biolegend), and IL9-PE (AH9R7; Biolegend). Peripheral blood mononuclear cells (PBMCs) were isolated with Lymphoprep Tubes (AXIS-SHIELD Poc AS, Oslo, Norway). For intracellular cytokine detection, PBMCs were stimulated with phorbol 12-myristate 13-acetate (10 ng/ml) and ionomycin (1 μg/ml) (both from Enzo Life Sciences, Plymouth Meeting, PA) in the presence of brefeldin A (Sigma-Aldrich, St. Louis, MO) for 4 h at 37°C in 5% CO_2, fixed, and permeabilised with BD FACS lysing solution and BD FACS permeabilising solution (BD Biosciences) according to the manufacturer's instructions. Data were acquired using a FACScan flow cytometer (MACSQuant Analyzer; Miltenyi Biotec) according to standard procedures for whole blood samples and PBMCs, as described previously [18,19]. Naïve T ($CCR7^+CD45RO^-$) cells, TCM ($CCR7^+CD45RO^+$), TEM ($CCR7^-CD45RA^-$), $CD8^+CD45RA^+$effector memory T ($CD8^+$ $CCR7^-CD45RA^+$) cells (TEMRA), regulatory T ($CD4^+CD25^{high}CD127^{low}$) cells (Treg), and suppressor precursor T ($CD28^-$) cells (Ts) were measured in all 23 patients in S1 Fig, while IFNγ-, IL17-, IL9-, and IL4-producing cells were analysed in 16 patients.

Statistical analysis

Demographic features between MS patients and HCs and between MS patients with and without relapse on fingolimod were compared by the Mann—Whitney U test, except for the sex ratio compared by Fisher's test. T cell counts and subset percentages between MS patients and HCs, between pre-treatment and post-treatment values in MS patients, and between MS patients with and without relapse on fingolimod were compared by the Mann—Whitney U test. For independent multiple comparisons, uncorrected p (p^{uncorr}) values were multiplied by the number of comparisons to calculate corrected p (p^{corr}) values (Bonferroni—Dunn's correction). Values of $p<0.05$ were considered to indicate statistical significance.

Results

Demographic features and treatment response to fingolimod

During fingolimod therapy, four patients had mild liver dysfunction and three patients had lymphopenia (less than 200 /μl), all of which recovered after cessation of the drug or symptomatic treatment. Also during fingolimod therapy, two patients had clinical relapses (both at 3 months after initiation of the drug) and six patients had new gadolinium-enhanced lesions on T1-weighted MRI or new/enlarging lesions on T2-weighted images at 6 or 12 months after initiation of the drug. These eight patients were classified as a relapsed group, while the remaining 15 patients were classified as a non-relapsed group. Although the relapsed patients had a shorter disease duration and higher Progression Index than the non-relapsed patients, there were no significant differences in any of the demographic features examined between the relapsed and non-relapsed patients (Table 1).

Comparisons of T cell subsets between MS patients at pre-treatment and HCs

There were no significant differences in the counts of lymphocytes, $CD4^+$T cells, and $CD8^+$T cells and the percentages of $CD4^+$T cells between the MS patients at pre-treatment and HCs, while the percentage of $CD8^+$T cells was significantly lower in MS patients than in HCs ($p<0.05$) (Fig 1A–1C). In addition, there were no significant differences in the percentages of naïve T cells, TCM, TEM, Treg, and Ts in $CD4^+$T cells and naïve T cells, TCM, TEM, TEMRA, and Ts in $CD8^+$T cells between the MS patients at pre-treatment and HCs (Fig 2A and 2B).

Alterations in T cell subsets during fingolimod therapy

The total lymphocyte counts were markedly decreased from 2 weeks until 12 months after initiation of fingolimod (Fig 1A), and the absolute counts and percentages of $CD4^+$T cells and the counts of $CD8^+$T cells were also decreased (Fig 1B and 1C). The absolute counts of TCM, naïve T cells, and TEM in both $CD4^+$T and $CD8^+$T cells and Treg in $CD4^+$T cells decreased significantly from 2 weeks to 12 months compared with the pre-treatment levels ($p<0.0001$ for all), as shown in S2 Fig.

In $CD4^+$T cells, the percentages of TCM and naïve T cells decreased significantly from 2 weeks to 12 months compared with the pre-treatment levels ($p<0.0001$ for all), while the TEM and Ts percentages increased from 2 weeks to 12 months compared with the pre-treatment levels ($p<0.0001$ for all) and the Treg percentages also increased from 2 weeks to 1 month ($p<0.05$) (Fig 2A). In $CD8^+$T cells, the percentages of naïve T cells and TCM decreased significantly from 2 weeks to 12 months compared with the pre-treatment levels, while the TEMRA and Ts percentages increased from 2 weeks to 12 months ($p<0.0001$ for all) (Fig 2B). However, the TEM percentages in $CD8^+$T cells showed no significant changes.

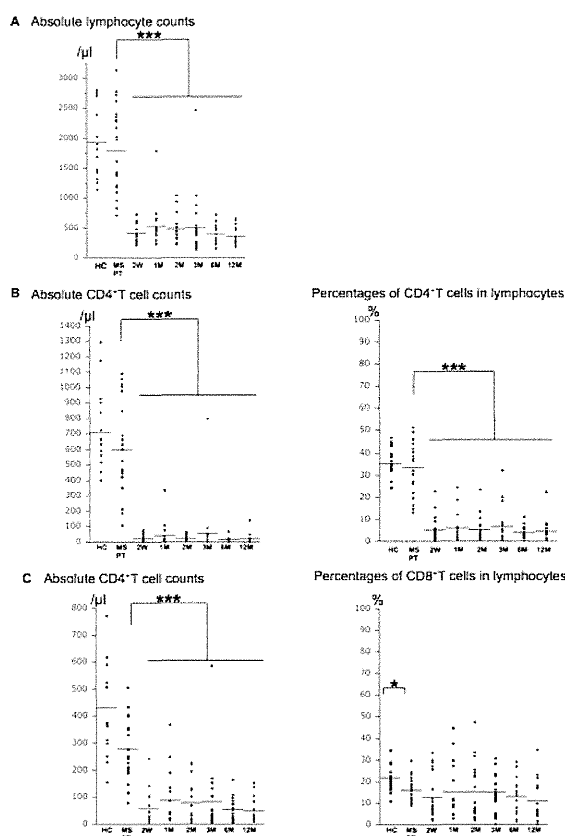

Fig 1. Counts of lymphocytes, CD4$^+$T, and CD8$^+$T cells are decreased from 2 weeks. Effects of fingolimod on peripheral blood lymphocyte counts (A) and absolute numbers and percentages of CD4$^+$T (B) and CD8$^+$T (C) cells in lymphocytes of healthy controls (HCs) and MS patients at pre-treatment (MS PT) and the indicated periods of fingolimod treatment. The numbers examined at each time point were: HC = 18, and MS PT = 23, 2W = 20, 1M = 17, 2M = 19, 3M = 23, 6M = 20, 12M = 18. The horizontal bars indicate the mean values. W = week; M = month. ***$p<0.0001$, *$p<0.05$.

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Comparisons of specific cytokine-producing T cell percentages between MS patients at pre-treatment and HCs

The percentages of IL17-, IL9-, and IL4-producing cells in CD4$^+$T cells were significantly higher in MS patients than in HCs ($p<0.01$ for all), and the percentages of IFNγ-, IL17-, IL9-, and IL4-producing cells in CD8$^+$T cells were also significantly higher in MS patients than in HCs ($p<0.05$, $p<0.01$, $p<0.01$, and $p<0.05$, respectively) (Fig 3). These trends were observed in IL17-, IL9-, and IL4-producing CD4$^+$T and CD8$^+$T cells in the MS patients, irrespective of whether they had IFNβ or corticosteroids within 3 months of initiation of fingolimod, as shown in S3 Fig. However, the increases in IFNγ-producing CD4$^+$T and CD8$^+$T cells at pre-treatment were only significant in the MS patients without IFNβ or corticosteroids.

Alterations in specific cytokine-producing T cell percentages during fingolimod therapy

The percentages of IFNγ-producing cells in both CD4$^+$T and CD8$^+$T cells and those of IL17-producing cells in CD4$^+$T cells increased transiently at 2 weeks after initiation of