Table 1
Clinical, allergological and MRI findings of patients with juvenile muscular atrophy of distal upper extremity

	Patient no.										
	1	2	3	4	5	6	7	8	9	10	11
Age at onset (years)	12	13	15	16	17	18	15	18	17	17	16
Age at examination (years)	13	15	22	17	19	19	15	20	20	22	19
Sex	F	F	M	M	M	M	M	M	M	M	M
Onset mode	Insidious	Insidious	Insidious	Insidious	Insidious	Insidious	Insidious	Insidious	Insidious	Insidious	Insidious
Muscle atrophy of distal upper extremity	R>L	R>L	R<L	R	R>L	R<L	R	R	R>L	R<L	R
Coexisting atopic/allergic disorders											
Bronchial asthma	+[a]	–	–	(+)[b]	–	–	–	–	–	–	(+)[b]
Allergic rhinitis	–	+	+	+	–	+	+	–	–	–	+
Pollinosis	–	–	+	–	–	–	–	+	+	+	+
Atopic dermatitis	–	–	–	–	–	–	(+)[b]	–	–	–	–
Others	+	–	–	–	–	+	+	+	+	+	–
Family history of atopic/allergic disorders	+	+	+	+	+	+	+	–	–	+	+
Peripheral blood eosinophil (%)[c]	7.8	7.6	2.7	4.5	6.7	5.2	5.3	4.4	14.2	2.0	8.3
Total serum IgE (IU/ml)	1874	1225	204	2400	150	220	242	<50	111	324	383
Allergen specific IgE (IU/ml)											
D. pteronyssinus	85.96	>100	9.19	>100	1.52	>100	6.75	–	–	–	44.02
D. farinae	90.66	>100	7.75	>100	2.63	>100	2.78	–	–	–	20.48
Cedar pollen	–	1.53	54.68	0.52	–	2.41	–	0.75	60.13	10.69	0.45
Soya bean	–	–	0.38	NE	–	–	–	–	1.19	0.45	–
Others	–	–	–	NE	–	–	–	–	Rice; 1.32	Wheat; 1.81	Wheat; 0.51
CSF cell count (/μl)	1	NE	NE	NE	NE	0	2	0	0	NE	0
CSF protein (mg/dl)	19	NE	NE	NE	NE	34	32	21	20	NE	17
Cervical MRI on flexion											
Cord flattening	+	+	+	+	+	+	+	+	+	+	–
Epidural venous dilatation	+	+	+	+	+	–	+	–	–	–	–

NE=not examined.
[a] Neurological symptoms developed gradually and were not related to asthma attacks.
[b] History of atopic/allergic disorders not present at the time of neurological illness is shown in parentheses.
[c] Eosinophil percentage higher than 4% is considered hypereosinophilia.

3.2. History of allergic disorders

The frequency of coexisting allergic disorders was significantly higher in patients with JMADUE than in the healthy control subjects (10/11 vs. 11/42, p=0.0002) (Fig. 1A). Of the patients, six (54.5%) had allergic rhinitis and five (45.5%) had pollinosis (Table 1), while in the normal control subjects, five (11.9%) had allergic rhinitis and three (7.1%) had pollinosis. The JMADUE group showed a significantly higher incidence of allergic rhinitis (p=0.0057) (Fig. 1B) and pollinosis (p=0.0064) (Fig. 1C). Two patients (patients 4 and 11) had a history of bronchial asthma that was not present at the time of their neurological illness. Although patient 1 had coexisting bronchial asthma when she developed JMADUE, the neurological symptoms developed gradually and were not related to the asthma attacks. None of the patients had coexisting atopic dermatitis but one (patient 7) had a history of atopic dermatitis that was not present at the time of their neurological illness. Nine of the eleven patients (81.8%) had a family history of allergic disorders in close relatives, and the frequency of a family history of allergic disorders was significantly greater in patients with JMADUE than in control subjects (5/42 (11.9%); p=0.0075) (Fig. 1D).

3.3. Allergological findings

Nine of the eleven patients (81.8%) showed mild to moderate eosinophilia (range 4.4% to 14.2%, normal<4.0%) in peripheral blood (Table 1). HyperIgEaemia was evident in five patients (45.5%), but the frequency of hyperIgEaemia was not significantly different between the patients with JMADUE and control subjects (12/42 (28.6%), p>0.1). All 11 patients had IgE specific for certain common allergens; 8 had IgE specific for two mite antigens, and 8 had IgE specific for cedar pollen. The frequency of mite antigen specific IgE was significantly higher in patients with JMADUE than in the control subjects (*D. pteronyssinus*, 8/11 vs. 14/42, p=0.0361; *D. farinae*, 8/11 vs. 14/42, p=0.0361) (Fig. 1E and F), but the frequency of cedar pollen specific IgE was not significantly different between the two groups (8/11 vs. 27/42, p>0.1).

3.4. Flow cytometric findings

The percentage of intracellular IFNγ^+IL-4$^-$CD4$^+$T cells in peripheral blood did not significantly differ between patients with JMADUE and control subjects (15.5±5.13 vs. 16.9±4.57) (Fig. 2A). The percentage of intracellular

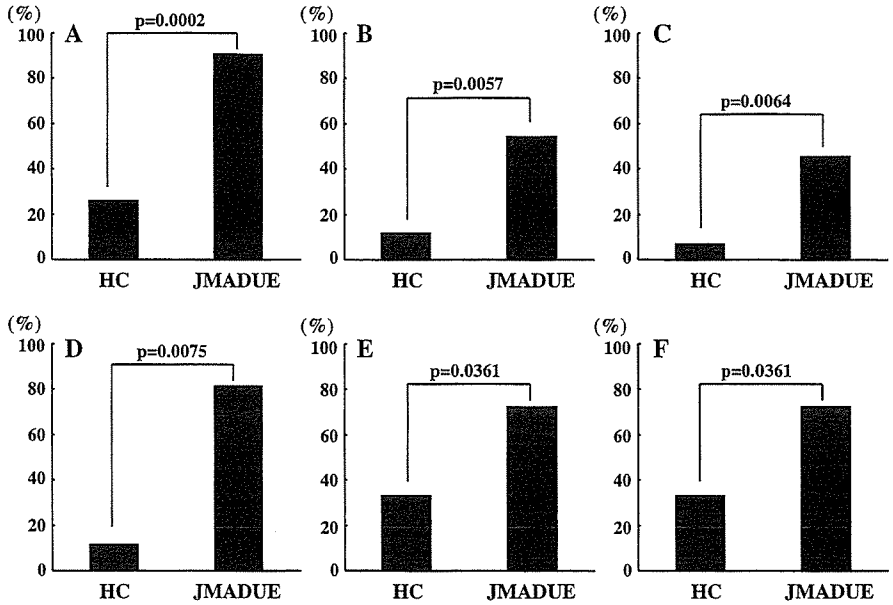

Fig. 1. Allergological features of juvenile muscular atrophy of the distal upper extremity. The frequencies of coexisting allergic disorders are shown: (A) total allergic disorders, (B) allergic rhinitis, (C) pollinosis, and a family history of allergic disorders (D). The frequency of mite antigen specific IgE is shown: (E) *D. pteronyssinus*, (F) *D. farinae*. The ordinate shows frequency in percentage. HC=healthy control subjects, JMADUE=juvenile muscular atrophy of the distal upper extremity.

IFNγ$^-$IL-4$^+$CD4$^+$T cells was significantly higher (2.73±0.32 vs. 2.02±0.68, p=0.0017) (Fig. 2B) in patients with JMADUE compared to control subjects, and hence the intracellular IFNγ/IL-4 ratio in CD4$^+$T cells was significantly reduced (5.67±1.82 vs. 8.92±2.95, p=0.002) (Fig. 2C). There was no significant change in the percentages of either IFNγ$^+$IL-4$^-$ or IFNγ$^-$IL-4$^+$CD8$^+$T cells between the two groups. Neither IL-5$^+$ nor IL-13$^+$ cell percentages showed any significant changes between the two groups in either CD4$^+$ or CD8$^+$T cell fractions.

4. Discussion

This is the first flow cytometric study of JMADUE to show a Th2 shift in the immune balance. Moreover, the present study on a greater scale confirmed a previous observation that the frequency of coexisting airway allergies such as allergic rhinitis and pollinosis, a family history of allergic disorders, and mite antigen specific IgE was significantly higher in patients with JMADUE compared to healthy control subjects and indicated a

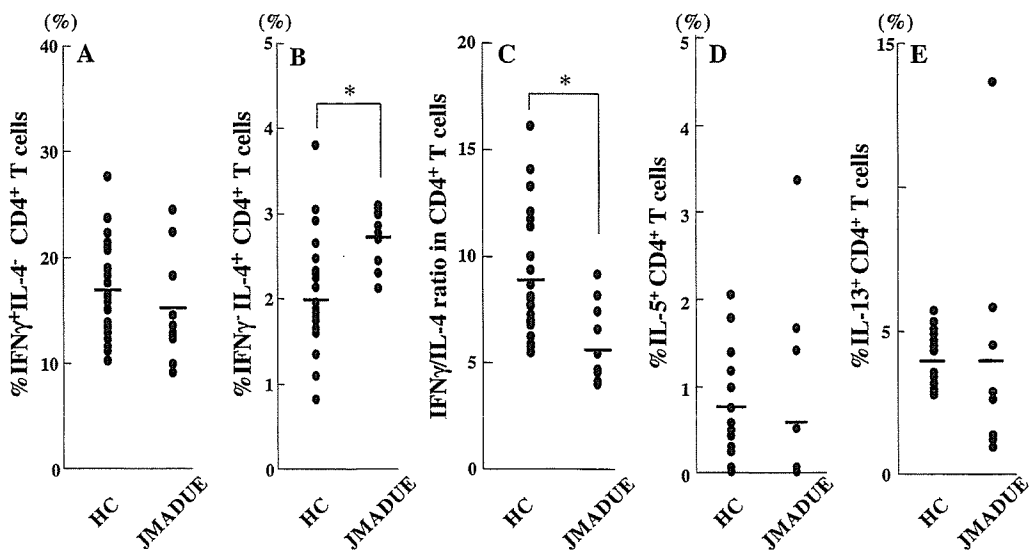

Fig. 2. Analysis of CD4$^+$T cell intracellular cytokine levels. The percentages of IFNγ$^+$IL-4$^-$ cells (A) and IFNγ$^-$IL-4$^+$ cells (B), IFNγ/IL-4 ratio (C) and the percentages of IL-5$^+$ cells (D) and IL-13$^+$ cells (E) in peripheral blood CD4$^+$T cells. *Statistically significant compared to healthy controls (p<0.01). The number of healthy controls in IL-5 and IL-13 assays is 16. HC=healthy control subjects, JMADUE=juvenile muscular atrophy of the distal upper extremity.

close association between atopic diathesis and JMADUE [4].

In this series, all patients displayed typical clinical features of JMADUE. Based on the radiological and neuropathological findings, repeated dynamic compression of the lower cervical cord followed by circulatory deficiencies may induce damage to the anterior horn cells, which are very vulnerable to ischemia [2,3]. However, neuroimaging showed that only 6 of the 11 patients exhibited flexion myelopathy, cervical cord flattening and epidural venous dilatation. Four patients showed mild cord flattening alone, and another had neither cord flattening nor forward displacement of the cervical dural sac. Other investigators have also failed to detect specific features of flexion myelopathy in some cases with this condition [9,10]. Furthermore, several investigators claim that such a forward displacement of the dural sac, and cord compression with neck flexion are even observed in normal people [9,11]. These findings indicate that JMADUE may be etiologically heterogeneous and that another mechanism other than flexion myelopathy may operate.

In the present study, we provide the first evidence of increases in the percentage of $IFN\gamma^{-}IL\text{-}4^{+}CD4^{+}T$ cells, and a reduction in the intracellular IFNγ/IL-4 ratio in $CD4^{+}T$ cells in JMADUE. Such a Th2 shift is consistent with hypereosinophilia and a heightened IgE response, which are frequently observed in this condition. It is well known that asthmatic amyotrophy (Hopkins syndrome), in which spinal motor neurons are affected, is also associated with atopic diathesis [12,13]. Thus airway allergy and related Th2 shift appear to contribute to cell damage of the spinal anterior horn of young people. On the other hand, we have reported occurrences of myelitis in patients with atopic dermatitis [14–16], naming it atopic myelitis. In this condition, the posterior column is preferentially involved. Although both JMADUE and atopic myelitis affect the cervical cord, involved sites on the axial plane of the spinal cord are distinct, i.e., anterior horns and posterior column, respectively. Therefore, both are considered to be distinct disease entities, yet both commonly have an allergic tendency and Th2 shift. It is interesting to note that atopic myelitis is preferentially associated with skin allergy whereas JMADUE and Hopkins syndrome are associated with airway allergy, thus suggesting a possibility that types of preceding atopic disorders may have some influence on the sites of involvement within the spinal cord.

Among the Th2 cytokines studied, IL-4, but not IL-5 or IL-13, was found to be upregulated in JMADUE. We previously reported that in atopic myelitis both IL-4 and IL-13 production was increased while IL-5 production was not elevated [7,17], and that opticospinal multiple sclerosis at relapse showed upregulation of IL-13 but neither IL-4 nor IL-5 [8]. The present findings together with the previous ones suggest that type 2 cytokine profiles are distinct among diseases affecting the spinal cord, and that in JMADUE IL-4 may play an important role. IL-4 is a key molecule for inducing a Th2 shift and allergic inflammation through a class switch from IgM to IgE [18], as well as stimulation for eotaxin synthesis [19]. Therefore, IgE-mediated activation of mast cells and/or eosinophils triggered by IL-4 [20] may be directly involved in spinal cord damage, or alternatively IL-4 may induce anti-neuronal autoantibodies that may damage spinal motor neurons. Human platelets can be activated by IgE and are therefore involved in the IgE-mediated effector mechanisms of allergic inflammation [21]. IgE-mediated activation of platelets causes platelet aggregation and histamine release, which may induce arterial spasm, as suggested by the increased incidence of circulatory deficiencies, such as cardiovascular diseases, in atopic patients [22–25]. It is possible that such a platelet-related disturbance of the circulatory system may also occur in JMADUE, in which IgE-mediated activation as well as repeated mechanical stress could be predisposing factors for platelet aggregation [26]. Plasma exchange, previously shown to be effective in some patients with JMADUE and airway allergy, may be beneficial by removing circulating factors, such as cytokines, IgE, autoantibodies and immune complexes. Another possibility is that allergic reaction and Th2 shift may be genetically linked to JMADUE, and have no direct involvement in anterior horn cell damage. However, as there have been no pathological studies of this disease during the early progressive stages, the nature of JMADUE remains unclear.

In summary, the numbers of Th2 cells were elevated in the peripheral blood of patients with JMADUE. The Th2 cytokine and IgE-mediated immune responses may contribute to the development of spinal cord damage in this condition, although comparison with patients with atopic diseases but without JMADUE will be required in the future. Further studies on cerebrospinal fluid cytokines are also called for to clarify the involvement of immunological processes in this condition.

Acknowledgements

This work was supported in part by a Neuroimmunological Disease Research Committee grant and a Research on Brain Science grant from the Ministry of Health and Welfare, Japan, and Grants-in-Aid 12557060 and 15590894 from the Ministry of Education, Science, Sports and Culture, Japan.

References

[1] Hirayama K, Tsubaki T, Toyokura Y, Okinaka S. Juvenile muscular atrophy of unilateral upper extremity. Neurology 1963;13:373–80.
[2] Hirayama K, Tokumaru Y. Cervical dural sac and spinal cord in juvenile muscular atrophy of distal upper extremity. Neurology 2000; 54:1922–6.
[3] Hirayama K, Tomonaga M, Kitano K, Yamada T, Kojima S, Arai K. Focal cervical poliopathy causing juvenile muscular atrophy of distal

upper extremity: a pathological study. J Neurol Neurosurg Psychiatry 1987;50:285–90.
[4] Kira J, Ochi H. Juvenile muscular atrophy of the distal upper limb (Hirayama disease) associated with atopy. J Neurol Neurosurg Psychiatry 2001;70:798–801.
[5] Ochi H, Murai H, Osoegawa M, Minohara M, Inaba S, Kira J. Juvenile muscular atrophy of distal upper extremity associated with airway allergy: two cases successfully treated by plasma exchange. J Neurol Sci 2003;206:109–14.
[6] Singh VK, Mehrotra S, Agarwal SS. The paradigm of Th1 and Th2 cytokines: its relevance to autoimmunity and allergy. Immunol Res 1999;20:147–61.
[7] Ochi H, Wu XM, Osoegawa M, Horiuchi I, Minohara M, Murai H, et al. Tc1/Tc2 and Th1/h2 balance in Asian and Western types of multiple sclerosis, HTLV-1-associated myelopathy/tropical spastic paraparesis and hyperIgEaemic myelitis. J Neuroimmunol 2001; 119:297–305.
[8] Ochi H, Osoegawa M, Wu X-M, Minohara M, Horiuchi I, Murai H, et al. Increased IL-13 but not IL-5 production by CD4-positive T cells and CD8-opsitive T cells in multiple sclerosis during relapse phase. J Neurol Sci 2002;201:45–51.
[9] Schroder R, Keller E, Flacke S, Schmidt S, Pohl C, Klockgether T, et al. MRI findings in Hirayama's disease: flexion-induced cervical myelopathy or intrinsic motor neuron disease? J Neurol 1999; 246:1069–74.
[10] Willeit J, Kiechl S, Kiechl-Kohlendorfer U, Golaszewski S, Peer S, Poewe W. Juvenile asymmetric segmental spinal muscular atrophy (Hirayama's disease): three cases without evidence of "flexion myelopathy". Acta Neurol Scand 2001;104:320–2.
[11] Robberecht W, Aguirre T, Van den Bosch L, Theys P, Nees H, Cassiman JJ, et al. Familial juvenile focal amyotrophy of the upper extremity (Hirayama disease). Superoxide dismutase 1 genotype and activity. Arch Neurol 1997;54:46–50.
[12] Mizuno Y, Komori S, Shigetomo R, Kurihara E, Tamagawa K, Komiya K. Poliomyelitis-like illness after acute asthma (Hopkins' syndrome). Brain Dev 1995;17:126–9.
[13] Horiuchi I, Yamasaki K, Osoegawa M, Ohyagi Y, Okayama A, Kurokawa T, et al. Acute myelitis after asthma attacks with onset after puberty. J Neurol Neurosurg Psychiatry 2000;68:665–8.
[14] Kira J, Yamasaki K, Kawano Y, Kobayashi T. Acute myelitis associated with hyperIgEemia and atopic dermatitis. J Neurol Sci 1997;148:199–203.
[15] Kira J, Kawano Y, Yamasaki K, Tobimatsu S. Acute myelitis with hyperIgEaemia and mite antigen specific IgE: atopic myelitis. J Neurol Neurosurg Psychiatry 1998;64:676–9.
[16] Kira J, Kawano Y, Horiuchi I, Yamada T, Imayama S, Furue M, et al. Clinical, immunological and MRI features of myelitis with atopic dermatitis (atopic myelitis). J Neurol Sci 1999;162:56–61.
[17] Ochi H, Osoegawa M, Murai H, Wu X-M, Taniwaki T, Kira J. Presence of IgE antibodies to bacterial superantigens and increased IL-13-producing T cells in myelitis with atopic diathesis. Int Arch Allergy Immunol 2004;134:41–8.
[18] Gauchat JF, Lebman DA, Coffman RL, Gascan H, de Vries JE. Structure and expression of germline ε transcripts in human B cells induced by interleukin 4 to switch to IgE production. J Exp Med 1990;172:463–73.
[19] Lezcano-Meza D, Dávila-Dávila B, Vega-Miranda A, Negrete-García LM, Teran LM. Interleukin (IL)-4 and to a lesser extent either IL-13 or interferon-gamma regulate the production of eotaxin-2/CCL24 in nasal polyps. Allergy 2003;58:1011–7.
[20] Katayama I, Tanei R, Yokozeki H, Nishioka K, Dohi Y. Induction of eczematous skin reaction in experimentally anti-DNP IgE antibody: possible implications for skin lesion formation in atopic dermatitis. Int Arch Allergy Appl Immunol 1990;93:148–54.
[21] Hasegawa S, Pawankar R, Suzuki K, Nakahata T, Furukawa S, Okumura K, et al. Functional expression of the high affinity receptor for IgE (FcepsilonRI) in human platelets and its' intracellular expression in human megakaryocytes. Blood 1999;93:2543–51.
[22] Knauer KA, Lichtenstein LM, Adkinson Jr NF, Fish JE. Platelet activation during antigen-induced airway reactions in asthmatic subjects. N Engl J Med 1981;304:1404–7.
[23] Criqui MH, Lee ER, Hamburger RN, Klauber MR, Coughlin SS. IgE and cardiovascular disease. Results from a population-based study. Am J Med 1987;82:964–8.
[24] Brunekreef B, Hoek G, Fischer P, Spieksma FT. Relation between airborne pollen concentrations and daily cardiovascular and respiratory-disease mortality. Lancet 2000;355:1517–8.
[25] Ginsburg R, Bristow MR, Kantrowitz N, Baim DS, Harrison DC. Histamine provocation of clinical coronary artery spasm: implications concerning pathogenesis of variant angina pectoris. Am Heart J 1981;102:819–22.
[26] Jesty J, Yin W, Perrotta P, Bluestein D. Platelet activation in a circulating flow loop: combined effects of shear stress and exposure time. Platelets 2003;14:143–9.

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Journal of the Neurological Sciences 228 (2005) 75–85

Journal of the Neurological Sciences

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Th1 shift in CIDP versus Th2 shift in vasculitic neuropathy in CSF

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Received 17 July 2004; received in revised form 1 October 2004; accepted 6 October 2004
Available online 12 November 2004

Abstract

To investigate the intra- and extracellular levels of various cytokines and chemokines in CSF in chronic inflammatory demyelinating polyneuropathy (CIDP) and vasculitic neuropathy (VN), 16 cytokines, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17, IFN-γ, TNF-α, G-CSF, MCP-1 and MIP-1β, were measured in CSF supernatant by a multiplexed fluorescent bead-based immunoassay and intracellular production of IFN-γ and IL-4 in CSF $CD4^+$ T cells were simultaneously measured by flow cytometry in 14 patients with CIDP, 8 patients with VN and 25 patients with other noninflammatory neurologic diseases (OND). In the CSF supernatant, a significant increase of IL-17, IL-8 and IL-6, and a significant decrease of IL-4, IL-5 and IL-7 levels were detected in pretreated CIDP as compared with OND. A significant increase of IL-6, IL-8 and IL-10 levels was found in pretreated VN. Both IL-17 and IL-8 levels correlated strongly with CSF protein levels in CIDP, although the correlation of IL-6 levels was weak. In CSF $CD4^+$ T cells, IFN-γ^+ IL-4^- cell percentages were markedly elevated in CIDP compared with OND, but not in VN, resulting in a significant increase of intracellular IFN-γ/IL-4 ratio in CIDP, even in the absence of CSF pleocytosis. The nonresponders to intravenous immunoglobulins (IVIGs) showed a significantly lower IFN-γ^- IL-4^+ $CD4^+$ T cell percentage, and tended to have a higher intracellular IFN-γ/IL-4 ratio than the responders in CSF. Marked upregulation of Th1 cytokine, IL-17, and downregulation of Th2 cytokines, together with infiltration of IFN-γ-producing $CD4^+$ T cells are useful markers for CIDP, while several Th2 cytokines are upregulated in VN in CSF.

Keywords: Th1; Th2; CSF; Cytokine; CIDP; Vasculitic neuropathy

1. Introduction

Chronic inflammatory demyelinating polyneuropathy (CIDP) is regarded as an autoimmune disease targeting peripheral nerve myelin, although its mechanism remains unknown. Immunohistochemical studies of biopsied sural nerves revealed that both $CD4^+$ and $CD8^+$ T cells infiltrated together along with activated macrophages but without either B cells or immunoglobulin deposition [1,2], suggesting a critical role for T cells. On the other hand, humoral immunity, such as immunoglobulins and immune complexes, is believed to be involved in vasculitic neuropathy (VN) [3].

Chemokines are crucial for recruiting T cells to inflammatory sites. Recent studies on chemokines in CIDP CSF revealed an increase in chemokines, attracting mainly Th1 cells, such as CXCL10 (IP-10) [4,5], which coincides with the highest expression of CXCR3. CXCR3 is a chemokine receptor for CXCL10 and is specific for Th1 cells among chemokine receptors in the invading T cells in biopsied sural nerves [4]. However, the significance of the increase in chemokines in CSF still remains unclear, as there is no CSF pleocytosis in CIDP, and none of the studies found any changes in the cellular composition of CSF in this condition.

Moreover, in CIDP CSF, cytokines such as IL-1α, IL-1β, IL-2, IL-6, IL-10, IFN-γ, TNF-α and monocyte colony-stimulating factor (M-CSF) were all found to be negative by an enzyme-linked immunosorbent assay (ELISA) [6], except for in one earlier study that showed an increase of

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0022-510X/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jns.2004.10.001

IL-6 [7]. However, a fluorescent bead-based immunoassay, with a wide dynamic range of standard curves, for multiple cytokines has become available, and requires only small amounts of materials for simultaneous measurements of multiple cytokines [8,9]. Therefore, the present study aimed to directly measure the intracellular cytokine production of $CD4^+$ T cells by flow cytometry, and various cytokine and chemokine levels of the CSF supernatant by multiplexed fluorescent bead-based immunoassay in CIDP and vasculitic neuropathy (VN). This was done to clarify the distinct immune balance in the CSF compartment. In addition, since CSF is considered to reflect events within the blood nerve barrier (BNB) more than in peripheral blood, we wanted to determine clinically useful CSF markers for each inflammatory neuropathy.

2. Materials and methods

2.1. Subjects

Fourteen patients with probable CIDP (9 males and 5 females, mean age±S.D.=60.1±17.2 years, range 28–80) based on the criteria of the American Academy of Neurology AIDS Task Force, were enrolled in this study [10]. In addition, 8 patients with untreated VN presented as mononeuritis multiplex (4 males and 4 females, mean age±S.D.=48.1±16.1 years, range 19–66) and 25 patients with other noninflammatory neurologic diseases (OND; 14 males and 11 females, mean age±S.D.=56.5±14.7 years, range 20–80) were also enrolled in the study. The OND group was composed of 14 patients with spinocerebellar degeneration, 2 with amyotrophic lateral sclerosis, 2 with cervical spondylosis, 2 with Alzheimer's disease and 1 each with Parkinson's disease, progressive supranuclear palsy, myelopathy, spinal cord infarction and epilepsy. The duration of CIDP ranged from 2 months to 24 years (mean±S.D.=6.69±8.95 years) at the time of lumbar puncture. Clinical courses were chronic progressive in 7, relapsing–remitting in 6 and monophasic in 1. Hughes grades [11] were 2 to 4 (mean±S.D.=3.21±0.89). All patients with CIDP showed motor dominant involvement and all but three showed symmetrical involvement. Eleven patients were treated by intravenous immunoglobulin (IVIG; 0.4g/kg/day) infusion for 5 days, and the six who improved by one or more than one grade on the Hughes scale after therapy were considered to be responders (Table 1).

2.2. Sample collection

At least 5 ml each of CSF and blood samples were obtained from all patients. In the case of CIDP, 26 CSF samples from 14 patients, 19 pretreatment and 7 posttreatment were obtained; all 8 CSF samples from the 8 VN patients were those from pretreatment. CSF samples were immediately centrifuged at 800 rpm/min at 4 °C for 5 min. The CSF cell counts in the samples used for the intracellular cytokine analysis were 2.0±1.0/μl (mean±S.D., range 1.0–4.0) in CIDP patients, 5.0±4.0/μl (mean±S.D., range 1.0–12.0) in VN patients and 1.0±0.5/μl (mean±S.D., range 1.0–2.0) in OND patients. CSF supernatant was kept under −70 °C until the cytokine assay.

2.3. Multiplexed fluorescent bead-based immunoassay

CSF supernatants were collected and analyzed simultaneously for 16 different cytokines and chemokines, namely, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17, IFN-γ, TNF-α, granulocyte colony-stimulating factor (G-CSF), monocyte chemoattractant protein 1 (MCP-1) and macrophage inflammatory protein 1β (MIP-

Table 1
Demographic features of patients with CIDP

Patient no.	Age at onset (year)	Age at examination (year)	Sex	Duration	Hughes grade at peak	CSF cells/protein (/μl, mg/dl)	Clinical course	Response to IVIG	Clinical symptoms	
									Predominant symptoms	Symmetrical involvement
1	58	65	M	7 y	2	2/148	relapsing	good	motor>sensory	+
2	72	72	F	2 m	2	1/28	monophasic	good	motor>sensory	−
3	27	28	M	1 y	4	2/87	relapsing	good	motor>>sensory	+
4	58	59	F	4 m	2	4/69	relapsing	good	motor>>sensory	+
5	75	77	F	3 y	4	1/253	CP	good	motor>sensory	+
6	49	53	M	4 y	4	1/39	CP	good	motor>>sensory	+
7	79	80	M	1 y	4	2/36	CP	poor	motor>>sensory	+
8	75	76	F	1 y	4	4/45	CP	poor	motor>>sensory	−
9	40	64	M	24 y	4	2/102	CP	poor	motor>sensory	+
10	60	80	M	20 y	4	2/68	relapsing	poor	motor>sensory	+
11	37	39	M	2 y	2	1/72	CP	poor	motor>sensory	+
12	12	36	M	24 y	3	1/30	relapsing	NE	motor	+
13	46	46	M	2 m	3	1/127	CP	NE	motor>sensory	+
14	61	67	F	6 y	3	0/30	relapsing	NE	motor	−

CIDP: chronic inflammatory demyelinating polyneuropathy, M: male, F: female, m: months, y: years, CSF: cerebrospinal fluid, IVIG: intravenous immunoglobulin, CP: chronic progressive, NE: not examined.

1β) using the Bio-Plex Cytokine Assay System (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions [8,9]. Briefly, 50 μl of each CSF supernatant and various concentrations of each cytokine standard (Bio-Rad) were added to 50 μl of antibody-conjugated beads (Bio-Rad) in a 96-well filter plate (Millipore, Billerica, MA). After a 30-min incubation, the plate was washed and 25 μl of a biotinylated antibody solution (Bio-Rad) was added to each well, followed by another 30-min incubation. The plate was then washed and 50 μl of streptavidin-conjugated PE (Bio-Rad) was added to each well and incubated for 10 min. Following a final wash, the contents of each well were resuspended in 125 μl of the assay buffer (Bio-Rad) and analyzed using a Bio-Plex Array Reader (Bio-Rad). The cytokine concentrations were calculated by reference to a standard curve for each cytokine derived using various concentrations of the cytokine standards (0.2, 0.78, 3.13, 12.5, 50, 200, 800 and 3200 pg/ml) assayed in the same manner as the CSF samples. The detection limit of each cytokine was determined by the recovery of the corresponding cytokine standard, and the lowest values showing more than 50% recovery were set as the lower detection limits. The lower detection limit for each cytokine was as follows: 0.2 pg/ml for IL-2, IL-4, IL-5, IL-7, IL-8, IL-10, IL-12 (p 70), IL-13, IL-17, IFN-γ and TNF-α, 0.78 pg/ml for IL-1β and IL-6, and 3.13 pg/ml for G-CSF, MCP-1 and MIP-1β. All samples were analyzed undiluted in duplicate. CSF supernatants used for the multiplexed fluorescent bead-based immunoassay were 26 CIDP (19 pre- and 7 posttreatment), 8 VN and 24 OND samples.

2.4. Intracellular cytokine analysis by flowcytometry

Each CSF supernatant was carefully removed and cell sediments were suspended in RPMI 1640 (Nipro, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS; Gibco BRL, Gaithersburg, MD; Lot#3217341S). This was followed by incubation with 25 ng/ml of phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO), 1.0 μg/ml of ionomycin (Sigma) and 10 μg/ml of Brefeldin A (BFA; Sigma) in a 24-well plate at 37 °C for 4 h under 5% CO_2. After washing with phosphate-buffered saline containing 0.1% bovine serum albumin (0.1% BSA-PBS), cells were stained with perCP-conjugated anti-CD4 monoclonal antibodies (Immunotech, Marseille, France) and incubated on ice in the dark for 15 min. Following another wash with 0.1% BSA-PBS, FACS permeabilizing solution (Becton Dickinson, San Jose, CA) was added and the cells were placed in the dark for 10 min. After two washes with 0.1% BSA-PBS, the cells were then stained with FITC-conjugated anti-IFN-γ (Immunotech) and PE-conjugated anti-IL-4 (Immunotech) for intracellular cytokine analysis, with mouse IgG2a-FITC (Immunotech) and IgG1-PE (Immunotech) as controls. After a 30-min incubation on ice in the dark, the percentages of intracellular IFN-γ-and IL-4-producing cells were immediately analyzed flowcytometrically using an Epics XL System II (Coulter, Hialeah, FL). Analysis gates were first set on lymphocytes according to the forward and side scatter properties and then set on $CD4^+$ lymphocytes. Cases with $CD4^+$ cell counts of less than 500 were discarded from the analysis to increase the reliability. For peripheral blood lymphocytes (PBL), intracellular cytokines were studied as described previously [12], using the same amounts of PMA, ionomycin and BFA, and the same monoclonal antibodies for staining. CSF cells taken from 10 patients with pretreated CIDP, 8 with VN at the pretreatment time and 12 OND patients, were used for intracellular cytokine analysis.

2.5. Statistical analysis

Statistical analyses were performed by the nonparametric Mann–Whitney *U* test to determine the significance between OND and each disease group. The difference between CSF and PBL in each group was analyzed by Wilcoxon signed rank test. In the CIDP group, the difference between responders and nonresponders was also analyzed by Mann–Whitney *U* test.

3. Results

3.1. Detection rates of each cytokine in CSF supernatant

The detection rate of IL-17 was significantly higher in pretreated CIDP patients compared with OND patients (19/19, 100% vs. 11/24, 45.8%, p=0.0004) and that of IL-10 was also significantly higher in VN patients (8/8, 100% vs. 11/24, 45.8%, p=0.0104), while that of IL-4 was significantly lower in pretreated and posttreated CIDP patients (10/19, 52.6% vs. 23/24, 95.8%, p=0.0022 and 3/7, 42.9% vs. 23/24, 95.8%, p=0.0051, respectively; Fig. 1). The detection rates of other cytokines were not significantly different between OND patients and each disease condition. IL-2 and IL-12 (p70) were not used for further statistical analyses because of low detection rates in CSF.

3.2. Comparison of cytokine levels in CSF supernatant among diseases

In CIDP patients, IL-17, IL-8 and IL-6 levels were significantly increased at the time of pretreatment as compared with OND levels (10.32±7.69 vs. 4.39±6.02 for the mean±S.D. and 10.33 vs. 0.19 for the median, p=0.0019; 31.17±19.99 vs. 14.23±9.20 and 25.50 vs. 11.32, p=0.0020; 42.94±27.82 vs. 62.89±179.80 and 30.29 vs. 21.55, p=0.0060, respectively; Fig. 2). After IVIG, the increase of IL-8 levels was still significant (31.62±12.26 vs. 14.23±9.29 and 32.00 vs. 11.32, p=0.0025) but not those of IL-17 (9.59±6.50 vs. 4.39±6.02 and 9.22 vs. 0.19) and IL-6 (44.94±26.26 vs. 62.89±179.77 and 31.95 vs. 21.55). In contrast, IL-4, IL-5

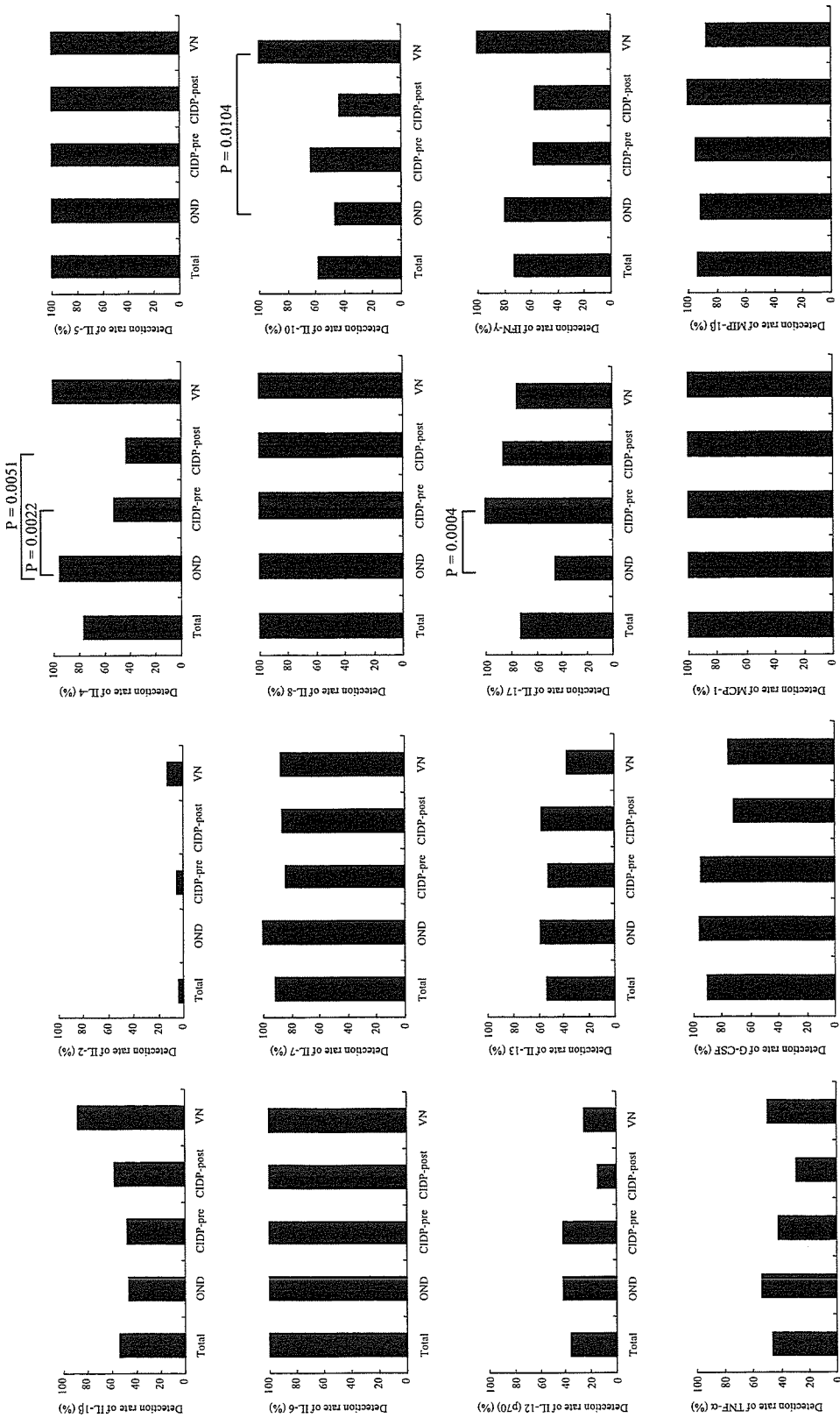

Fig. 1. Detection rates of each cytokine in CSF supernatant by multiplexed fluorescent bead-based immunoassay. The total of 58 patients are 24 in OND, 19 in pretreated CIDP, 7 in posttreated CIDP and 8 in VN.

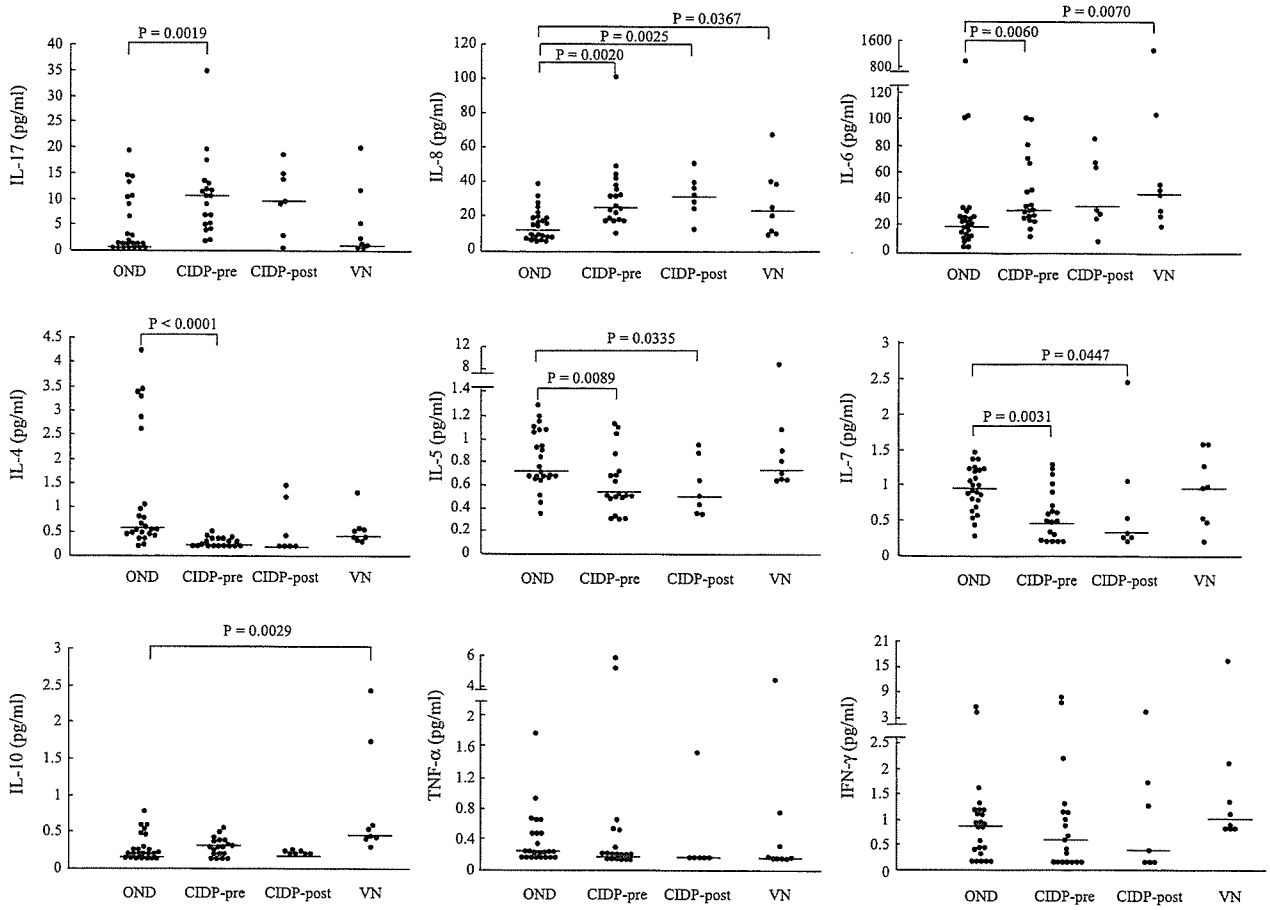

Fig. 2. Cytokine levels in the CSF supernatant in OND, CIDP and VN by multiplexed fluorescent bead-based immunoassay. CIDP-pre=CIDP at the pretreatment. CIDP-post=CIDP after the IVIG (6 patients were responders and 5 nonresponders). IL-1β, IL-13, G-CSF, MCP-1 and MIP-1β are not shown in the figure, but mean±S.D. and median values of these cytokines are given in the text. IL-2 and IL-12 (p70) are not shown due to the low detection frequency in CSF.

and IL-7 levels were significantly decreased at pretreatment (0.27±0.09 vs. 1.22±1.26 and 0.22 vs. 0.55, $p<0.0001$, 0.62±0.26 vs. 0.82±0.25 and 0.57 vs. 0.74, $p=0.0089$, and 0.58±0.36 vs. 0.94±0.31 and 0.48 vs. 0.95, $p=0.0031$, respectively). At the time of posttreatment, decreases of IL-5 and IL-7 levels were still significant (0.59±0.24 vs. 0.82±0.25 and 0.52 vs. 0.74, $p=0.0335$, and 0.72±0.81 vs. 0.94±0.31 and 0.31 vs. 0.95, $p=0.0447$, respectively) but this was not the case for IL-4 levels (0.54±0.53 vs. 1.22±1.26 and 0.19 vs. 0.55). None of the other cytokines or chemokines showed any significant changes at either the pre- or posttreatment times. The concentrations of the other cytokines and chemokines, which did not show any significant changes, were as follows in the pretreatment CIDP, posttreatment CIDP and OND patients: IL-1β (pg/ml), 1.55±1.11 (mean±S.D.) and 0.77 (median), 2.25±2.56 and 0.93, and 1.36±0.73 and 0.77; IL-13 (pg/ml), 0.77±1.05 and 0.20, 0.9±1.55 and 0.34, and 0.65±0.76 and 0.24; IFN-γ (pg/ml), 1.47±2.55 and 0.53, 1.16±1.47 and 0.43, and 1.05±1.18 and 0.81; G-CSF (pg/ml), 33.24±29.61 and 21.11, 14.74±9.70 and 18.20, and 25.35±21.50 and 19.85; MCP-1 (pg/ml), 219.05±154.72 and 169.71, 145.97±89.18 and 99.55, and 174.3±69.31 and 160.32; MIP-1β (pg/ml), 11.05±6.3 and 10.80, 13.31±7.9 and 12.79, and 16±13.85 and 10.28; IL-10, (pg/ml) 0.29±0.11 and 0.29, 0.21±0.02 and 0.19, and 0.30±0.17 and 0.19; TNF-α (pg/ml) 0.80±1.64 and 0.19, 0.42±0.46 and 0.19, and 0.36±0.32 and 0.20, respectively.

In contrast, VN patients showed a significant increase of IL-6, IL-8 and IL-10 levels as compared with OND patients at the time of pretreatment (208.54±457.09 vs. 62.89±179.77 and 43.41 vs. 21.55, $p=0.0070$, 27.52±20.30 vs. 14.23±9.29 and 22.53 vs. 11.32, $p=0.0367$, and 0.85±0.78 vs. 0.30±0.17 and 0.48 vs. 0.19, $p=0.0029$, respectively). There were no significant changes in concentration of any of the other cytokines and chemokines in VN patients, and the results are as follows: IL-1β (pg/ml), 1.63±1.52 (mean±S.D.) and 1.16 (median); IL-4 (pg/ml), 0.53±0.33 and 0.44; IL-5 (pg/ml), 1.80±2.91 and 0.75; IL-7 (pg/ml), 0.94±0.52 and 0.95; IL-13 (pg/ml), 0.64±0.75 and 0.19; IL-17 (pg/ml), 5.07±7.07 and 1.58; IFN-γ (pg/ml), 2.98±5.45 and 1.0; TNF-α (pg/ml), 0.77±1.41 and 0.20; G-CSF (pg/ml), 30.52±22.55 and 35.02; MCP-1 (pg/ml), 182.22±96.40 and 169.82; MIP-1β (pg/ml), 9.47±5.04 and 6.67.

3.3. Comparison of intracellular cytokine production between CSF cells and PBL

Representative staining patterns for the cytokines in each group are shown in Fig. 3. CSF cells showed a significantly higher proportion of IFN-γ^+ IL-4$^-$ CD4$^+$ T cells than in PBL in CIDP patients (p=0.0051), VN (p=0.0117) and OND patients (p=0.0022; Fig. 4). IFN-γ^- IL-4$^+$ CD4$^+$ T cell percentages were significantly lower in CSF cells than in PBL in VN patients (p=0.0421) and OND patients (p=0.0342), and also tended to be lower in CIDP patients (p=0.0745). Thus, the intracellular IFN-γ/IL-4 ratio (IFN-γ^+ IL-4$^-$ CD4$^+$ T cell percentage divided by IFN-γ^- IL-4$^+$ CD4$^+$ T cell percentage) was significantly higher in CSF cells than in PBL in CIDP patients (p=0.0051), VN (p=0.0117) and OND patients (p=0.0029). IFN-γ^+ IL-4$^+$ cell percentages were significantly greater in CSF cells than in PBL in CIDP patients (p=0.0284), VN (p=0.0117) and OND patients (p=0.0229).

3.4. Comparison of intracellular cytokine production of CSF cells and PBL among diseases

In CSF cells, IFN-γ^+ IL-4$^-$ cell percentages in CIDP patients showed clustering in the high ranges, while those in VN patients were distributed over a wide range (Fig. 4). Thus, IFN-γ^+ IL-4$^-$ cell percentages were markedly increased in CIDP compared with OND patients (p=0.0008), but not in

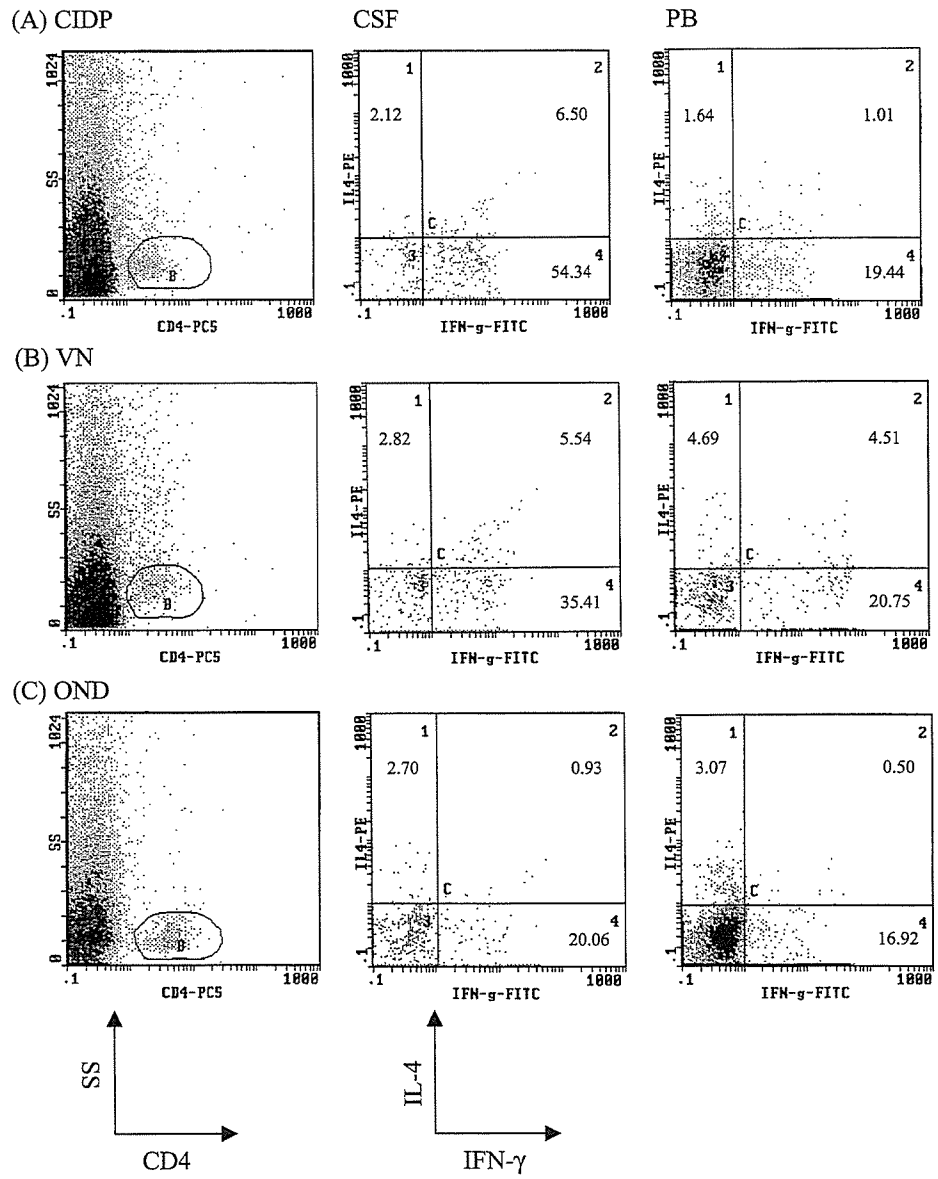

Fig. 3. Representative staining patterns of the intracellular cytokines in CSF CD4$^+$ T cells and peripheral blood (PB) CD4$^+$ T cells from CIDP, VN and OND patients. (A) A 59-year-old patient with CIDP. (B) A 51-year-old patient with VN. (C) A 52-year-old patient with amyotrophic lateral sclerosis. Note the increase in IFN-γ^+ IL-4$^-$ CD4$^+$ T cells in the CIDP CSF cells compared with OND.

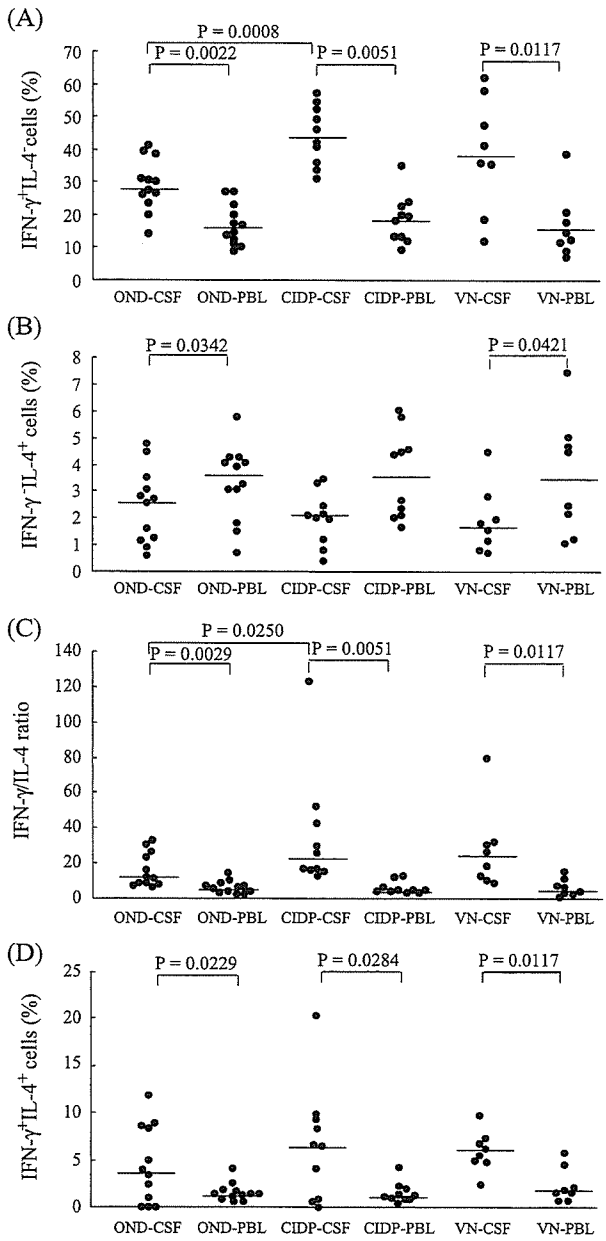

Fig. 4. Intracellular cytokine production patterns of CSF $CD4^+$ T cells in CIDP, VN and OND. (A) IFN-γ^+ IL-4^- $CD4^+$ T cell percentages. (B) IFN-γ^- IL-4^+ $CD4^+$ T cell percentages. (C) Intracellular IFN-γ/IL-4 ratios in $CD4^+$ T cells. (D) IFN-γ^+ IL-4^+ $CD4^+$ T cell percentages.

VN patients, while in PBL, the percentages did not differ significantly among the diseases. IFN-γ^- IL-4^+ cell percentages were not significantly different among the groups in either CSF cells or PBL. Thus, the intracellular IFN-γ/IL-4 ratio was significantly increased in CSF cells in CIDP compared with OND patients (p=0.0250). The ratios in CSF in VN patients, and in PBL in either CIDP or VN patients were not significantly different compared with OND patients. The proportions of IFN-γ^+ IL-4^+ cells in either CSF cells or PBL were not significantly different among the groups.

3.5. Correlation between clinical parameters and intra- and extracellular cytokine levels in CIDP patients

Both IL-17 and IL-8 in CSF supernatant showed significant positive correlation with CSF protein concentrations (r=0.468, p=0.0027 and r=0.579, p=0.0022, respectively; Fig. 5). CIDP patients with increased CSF protein levels at examination had significantly higher levels

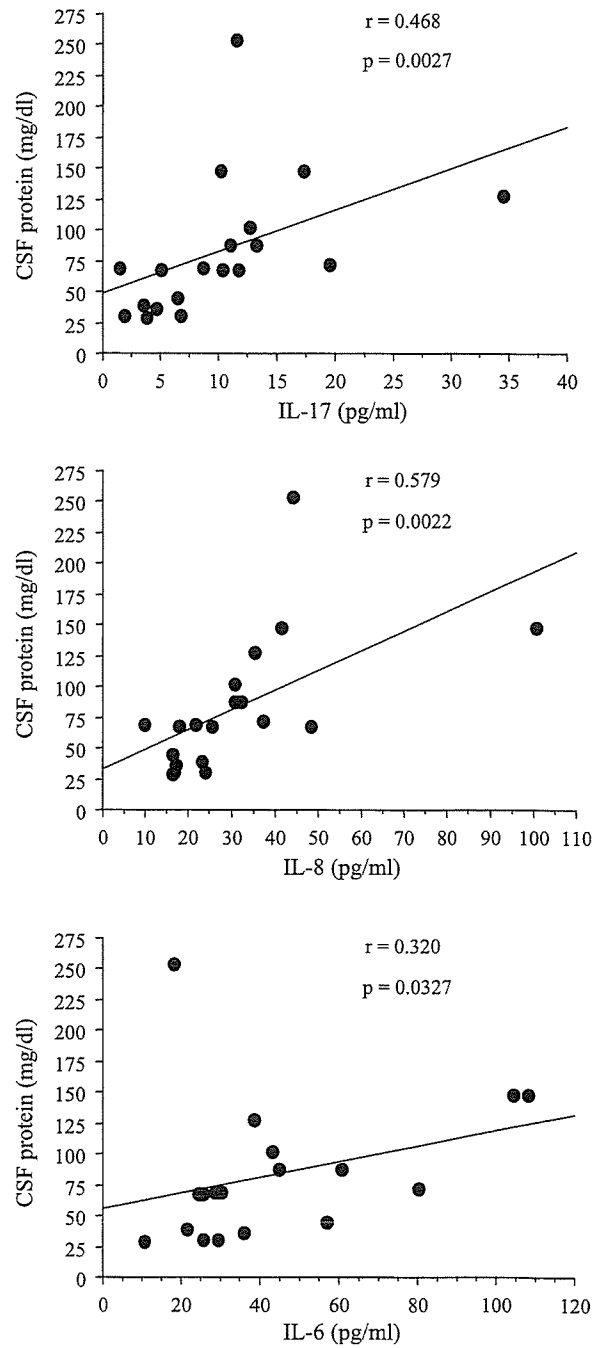

Fig. 5. Correlation between IL-17, IL-8 and IL-6 levels in CSF supernatant and CSF protein concentration. Both IL-17 and IL-8 in CSF supernatant showed a strong positive correlation with CSF protein concentration, while IL-6 showed a weak correlation with CSF protein levels.

of IL-17 and IL-8 than those without the protein increase (12.5±7.83 vs. 4.21±1.75 for the mean±S.D. and 11.39 vs. 3.94 for the median, p=0.0095 in IL-17, and 35.31±21.88 vs. 19.57±3.79 and 31.88 vs. 17.33, p=0.0464 in IL-8). IL-6 levels also showed a weak correlation with CSF protein levels (r=0.320, p=0.0327), and IL-6 levels in CSF tended to be higher in CIDP patients with increased CSF protein levels than in those without the increase (49.4±29.54 vs. 24.83±9.52 and 41.16 vs. 25.85, p=0.0641). In CSF cells of CIDP patients, IVIG nonresponders showed significantly lower IFN-γ$^-$ IL-4$^+$ CD4$^+$ T cell percentages in CSF at pretreatment than responders (p=0.0105), and the intracellular IFN-γ/IL-4 ratio also tended to be higher in nonresponders than responders (p=0.0811; Fig. 6). No other clinical parameters, such as age at onset or at examination, duration of disease, sex, clinical course and Hughes grade, had any significant correlation with intra- and extracellular cytokine amounts in CSF.

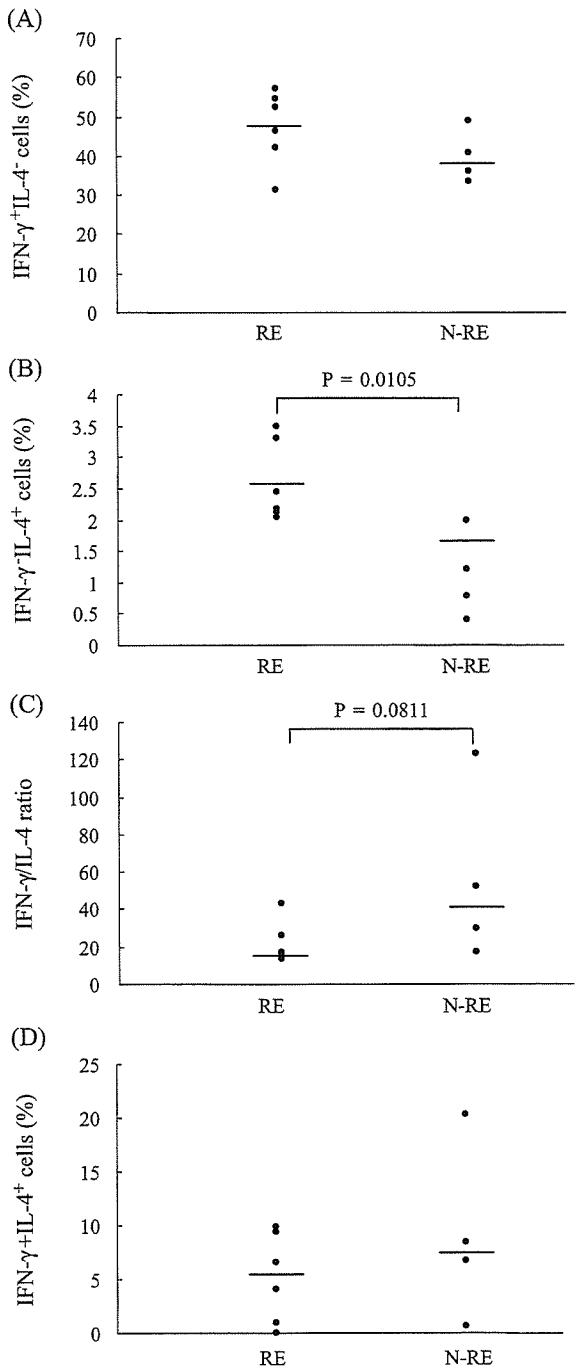

Fig. 6. Comparison of intracellular cytokine production patterns between IVIG responders (RE) and nonresponders (N-RE) in CIDP. (A) IFN-γ$^+$ IL-4$^-$ CD4$^+$ T cell percentages. (B) IFN-γ$^-$IL-4$^+$ CD4$^+$ T cell percentages. (C) Intracellular IFN-γ/IL-4 ratios in CD4$^+$ T cells. (D) IFN-γ$^+$ IL-4$^+$ CD4$^+$ T cell percentages.

4. Discussion

The present study is the first to demonstrate changes in intracellular cytokine production in CSF cells and changes in secreted cytokine levels in CSF supernatant, by a combined intra- and extracellular cytokine analysis in CIDP and VN. Our method successfully measured the intracellular cytokine production even in the absence of CSF pleocytosis. Cytokine assessment at the cellular level in the CSF has been difficult because of the extreme fragility of CSF cells and their limited numbers. In the current study, even in the absence of CSF pleocytosis, CIDP patients were found to have a marked increase in IFN-γ-producing CD4$^+$ T cells and thus a greater Th1 shift in the CSF compartment than OND patients, which coincided with upregulation of type 1 cytokine, IL-17, and downregulation of type 2 cytokines, such as IL-4 and IL-5. In contrast, VN patients showed upregulation of type 2 cytokines, such as IL-6 and IL-10.

Among the cytokines that were elevated in the CSF supernatant in the pretreated patients, IL-17 was upregulated in CIDP patients but not in VN patients. In addition, IL-10 was elevated in VN patients but not in CIDP patients, whereas both IL-6 and IL-8 levels were upregulated in CIDP patients as well as VN patients, suggesting unique roles for IL-17 in CIDP and IL-10 in VN. IL-17 is a potent proinflammatory cytokine produced by a subset of memory CD4$^+$ T cells, mainly Th1 type, on activation [13–15]. IL-17 triggers the local production of downstream cytokines and chemokines, such as IL-6, IL-8, MCP-1 and G-CSF, from a wide variety of cells [13,15,16]. It is possible that increase of IL-6 and IL-8 found in the present study may be triggered by IL-17. In addition, IL-17 stimulates the production of matrix metalloproteinases [17,18], iNOS, NO [19,20] and PGE2 [13,21], which enhance the local inflammatory environment; as shown in rheumatoid arthritis (RA) where high levels of IL-17 in synovial fluid contribute to inflammatory joint destruction [22,23]. In the present study, both IL-17 and IL-8 levels positively correlated with CSF protein concentrations in CIDP patients, which indicates that an increase of those cytokines/chemokines is related to the severity of inflammation in the spinal roots. Both IL-6 and IL-8 are proinflammatory cytokines and contribute to exacerbate various

autoimmune inflammatory diseases, such as rheumatoid arthritis (RA) [24], systemic lupus erythematosus (SLE) [25], ulcerative colitis [26], Crohn's disease [27] and psoriasis [24]. IL-6 induces an acute phase response, activates monocytes [28] and induces other chemokines [29], such as MIP-2, and adhesion molecules [30]; while IL-8 recruits granulocytes and T cells to the local inflammatory sites [31,32]. Therefore, increased IL-6 and IL-8 responses could also contribute to enhance local inflammation in CIDP.

In contrast, type 2 cytokines, IL-4 and IL-5, were downregulated in CSF supernatant only in CIDP patients. IL-7, which was originally described as a growth and differentiation factor for precursor B cells [33] and was later shown to prime human naïve $CD4^+$ T cells for IFN-γ as well as IL-4 [34], was downregulated in CIDP CSF in the present study. The underlying disease process of CIDP appears to cause this cytokine to behave similarly to type 2 cytokines. This finding is similar to those in other organ-specific autoimmune diseases, such as bullous pemphigoid, where IL-7 is downregulated more in the blister fluid than in the sera [35].

IVIG treatment has been shown to be effective in CIDP and can shift the immune balance from the Th1 to the Th2 side [36,37]. Therefore, the beneficial effects of giving IVIG in CIDP are at least partly attributable to the correction of the pathogenic Th1 shift. However, since the nonresponders to IVIG showed significantly lower IFN-γ^- IL-4^+ $CD4^+$ T cells than responders, IVIG does not appear able to overcome the disease process in CIDP patients with markedly depressed Th2 cells in CSF.

Mathey et al. [38] showed that mRNA expression of Th1 cytokines, such as IFN-γ, TNF-α and IL-2, was upregulated in the biopsied sural nerves from CIDP patients. However, in CSF supernatant, IL-2 was barely detectable in any of the diseases examined, and the increases of TNF-α and IFN-γ concentrations were not statistically significant. Although one ELISA study has demonstrated an increase of TNF-α concentrations in CIDP sera in a fraction of patients with grave disability [39], in the present study, only a minority of CIDP patients with grave disease (Hughes grade 4) had very high TNF-α concentrations in CSF. This observation might be explained by the very short half life of TNF-α, i.e., 30 min [40], or alternatively, a distinct subset of CIDP patients may preferentially produce TNF-α. For IFN-γ, intracellular cytokine analysis is superior to extracellular cytokine measurement; probably because intracellular cytokine analysis measures the preprogrammed potential of cytokine production in each cell directly, and thus the amounts are not diluted by other factors, such as large volumes of continually replacing CSF.

Although we previously reported that Th2 cells are also increased in peripheral blood in some CIDP patients by intracellular cytokine analysis [41], Th1 shift is evident in CSF cells. It is possible that Th1 cells move from peripheral blood to nerves beyond the BNB, and CSF cells more sensitively reflect the pathological process occurring within the BNB in CIDP.

In the present study, IL-6, IL-8 and IL-10 levels were found to be upregulated in CSF supernatant in VN patients; a group in which CSF cytokine changes have never been reported. In addition, acting as a proinflammatory cytokine, IL-6 increases fibrinogen amounts [42] and platelet number [43], and increases blood viscosity through the induction of hepatic acute phase response [24]. Therefore, it is possible that an increase of IL-6 exacerbates vasculitis-related ischemia of the peripheral nerves. Furthermore, both IL-6 and IL-10 can enhance autoantibody production [24,25,44–46]. Although the increase of IL-10 in VN could be the result of the host's efforts to counteract inflammation since IL-10 works as anti-inflammatory cytokine [47], IL-10 itself is regarded as an exacerbating factor in vasculitic disorders, such as SLE [25,48], RA [46], Sjögren's syndrome [44] and Wegener's granulomatosis [45], through enhanced autoantibody production. As immunoglobulins, complements and immune complexes are deposited in the vessel wall in VN [3,49], enhanced IL-6 and IL-10 responses in CSF may act as exacerbating factors in this condition.

Since no increase of intracellular IFN-γ/IL-4 ratio in CSF cells was observed in VN patients, the commitment of Th1 cells may be less important in VN than in CIDP. However, intracellular IFN-γ^+ IL-4^- $CD4^+$ cell percentages in VN CSF were distributed over a wide range, which is in contrast to the clustering observed in the high ranges in CIDP patients. This indicates the heterogeneous nature of VN and further investigation of CSF intracellular cytokine analyses together with further subclassification of VN is required.

In conclusion, our combined multiplexed fluorescent bead-based immunoassay and intracellular cytokine analysis of CSF successfully demonstrated the upregulation of Th1 and downregulation of Th2 cytokines, increased IFN-γ producing $CD4^+$ T cells in CIDP patients and the upregulation of some Th2 cytokines in VN patients. Thus, we propose that IL-4, IL-5, IL-6, IL-7, IL-8, IL-10 and IL-17 are clinically useful sets of cytokines for CSF immune monitoring by multiplexed fluorescent bead-based immunoassay.

Acknowledgements

This work was supported in part by a Neuroimmunological Disease Research Committee grant, a Research on Brain Science grant from the Ministry of Health and Welfare, Japan, and Grants 12470142, 12557060 and 12877097 from the Ministry of Education, Science, Sports and Culture, Japan.

References

[1] Matsumuro K, Izumo S, Umehara F, Osame M. Chronic inflammatory demyelinating polyneuropathy: histological and immunopathological studies on biopsied sural nerves. J Neurol Sci 1994;127:170–8.
[2] Rizzuto N, Morbin M, Cavallaro T, Ferrari S, Fallahi M, Galiazzo Rizzuto S. Focal lesions area feature of chronic inflammatory

demyelinating polyneuropathy (CIDP). Acta Neuropathol (Berl) 1998; 96:603–9.
[3] Davies L, Spies JM, Pollard JD, McLeod JG. Vasculitis confined to peripheral nerves. Brain 1996;119:1441–8.
[4] Kieseier BC, Tani M, Mahad D, Oka N, Ho T, Woodroofe N, et al. Chemokines and chemokine receptors in inflammatory demyelinating neuropathies: a central role for IP-10. Brain 2002;125:823–34.
[5] Mahad DJ, Howell SJL, Woodroofe MN. Expression of chemokines in cerebrospinal fluid and serum of patients with chronic inflammatory demyelinating polyneuropathy. J Neurol Neurosurg Psychiatry 2002; 73:320–3.
[6] Sivieri S, Ferrarini AM, Lolli F, Matà S, Pinto F, Tavolato B, et al. Cytokine pattern in the cerebrospinal fluid from patients with GBS and CIDP. J Neurol Sci 1997;147:93–5.
[7] Maimone D, Annunziata P, Simone IL, Livrea P, Guazzi GC. Interleukin-6 levels in the cerebrospinal fluid and serum of patients with Guillain–Barré syndrome and chronic inflammatory demyelinating polyradiculoneuropathy. J Neuroimmunol 1993;47:55–61.
[8] Vignali DAA. Multiplexed particle-based flow cytometric assays. J Immunol Methods 2000;243:243–55.
[9] de Jager W, te Velthuis H, Prakken BJ, Kuis W, Rijkers GT. Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells. Clin Diagn Lab Immunol 2003;10:133–9.
[10] Report from an Ad Hoc Subcommittee of the American Academy of Neurology AIDS Task Force. Research criteria for diagnosis of chronic inflammatory demyelinating polyneuropathy (CIDP). Neurology 1991;41:617–8.
[11] Hughes RAC, Newsom-Davis JM, Perkin GD, Pierce JM. Controlled trial prednisolone in acute polyneuropathy. Lancet 1978;2:750–3.
[12] Horiuchi I, Kawano Y, Yamasaki K, Minohara M, Furue M, Taniwaki T, et al. Th1 dominance in HAM/TSP and the optico-spinal form of multiple sclerosis versus Th2 dominance in mite antigen-specific IgE myelitis. J Neurol Sci 2000;172:17–24.
[13] Fossiez F, Djossou O, Chomarat P, Flores-Romo L, Ait-Yahia S, Maat C, et al. T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. J Exp Med 1996;183: 2593–603.
[14] Schwarzenberger P, Huang W, Ye P, Oliver P, Manuel M, Zhang Z, et al. Requirement of endogenous stem cell factor and granulocyte-colony-stimulating factor for IL-17-mediated granulopoiesis. J Immunol 2000;164:4783–9.
[15] Aggarwal S, Gurney AL. IL-17: prototype member of an emerging cytokine family. J Leukoc Biol 2002;71:1–8.
[16] Hwang S-Y, Kim J-Y, Kim K-W, Park M-K, Moon Y, Kim W-U. IL-17 induces production of IL-6 and IL-8 in rheumatoid arthritis synovial fibroblasts via NF-κB- and PI3-kinase/Akt-dependent pathways. Arthritis Res Ther 2004;6:R120–8.
[17] Jovanovic DV, Martel-Pelletier J, Di Battista JA, Mineau F, Jolicoeur F-C, Benderdour M, et al. Stimulation of 92-kd gelatinase (matrix metalloproteinase 9) production by interleukin-17 in human monocyte/macrophages: a possible role in rheumatoid arthritis. Arthritis Rheum 2000;43:1134–44.
[18] Prause O, Bozinovski S, Anderson GP, Lindén A. Increased matrix metalloproteinase-9 concentration and activity after stimulation with interleukin-17 in mouse airways. Thorax 2004;59:313–7.
[19] Trajkovic V, Stosic-Grujicic S, Samardzic T, Markovic M, Miljkovic D, Ramic Z, et al. Interleukin-17 stimulates inducible nitric oxide synthase activation in rodent astrocytes. J Neuroimmunol 2001;119:183–91.
[20] Miljkovic D, Trajkovic V. Inducible nitric oxide synthase activation by interleukin-17. Cytokine Growth Factor Rev 2004;15:21–32.
[21] Kotake S, Udagawa N, Takahashi N, Matsuzaki K, Itoh K, Ishiyama S, et al. IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis. J Clin Invest 1999;103:1345–52.
[22] Ziolkowska M, Koc A, Luszczykiewicz G, Ksiezopolska-Pietrzak K, Klimczak E, Chwalinska-Sadowska H, et al. High levels of IL-17 in rheumatoid arthritis patients: IL-15 triggers in vitro IL-17 production via cyclosporin A-sensitive mechanism. J Immunol 2000;164:2832–8.
[23] Dudler J, Renggli-Zulliger N, Busso N, Lotz M, So A. Effect of interleukin 17 on proteoglycan degradation in murine knee joints. Ann Rheum Dis 2000;59:529–32.
[24] Ishihara K, Hirano T. IL-6 in autoimmune disease and chronic inflammatory proliferative disease. Cytokine Growth Factor Rev 2002;13:357–68.
[25] Cross JT, Benton HP. The roles of interleukin-6 and interleukin-10 in B cell hyperactivity in systemic lupus erythematosus. Inflamm Res 1999;48:255–61.
[26] Larsen TB, Nieslsen JN, Fredholm L, Lund ED, Brandslund I, Munkholm P, et al. Platelets and anticoagulant capacity in patients with inflammatory bowel disease. Pathophysiol Haemost Thromb 2002;32:92–6.
[27] Ito H. IL-6 and Crohn's disease. Curr Drug Targets Inflamm Allergy 2003;2:125–30.
[28] Sebbag M, Parry SL, Brennan FM, Feldmann M. Cytokine stimulation of T lymphocytes regulates their capacity to induce monocyte production of tumor necrosis factor-alpha, but not interleukin-10: possible relevance to pathophysiology of rheumatoid arthritis. Eur J Immunol 1997;27:624–32.
[29] McLoughlin RM, Hurst SM, Nowell MA, Harris DA, Horiuchi S, Morgan LW, et al. Differential regulation of neutrophil-activating chemokines by IL-6 and its soluble receptor isoforms. J Immunol 2004;172:5676–83.
[30] Dahlman-Ghozlan K, Ortonne J-P, Heilborn JD, Stephansson E. Altered tissue expression pattern of cell adhesion molecules, ICAM-1, E-selectin and VCAM-1, in bullous pemphigoid during methotrexate therapy. Exp Dermatol 2004;13:65–9.
[31] Chertov O, Michiel DF, Xu L, Wang JM, Tani K, Murphy WJ, et al. Identification of defensin-1, defensin-2, and CAP37/azurocidin as T-cell chemoattractant proteins released from interleukin-8-stimulated neutrophils. J Biol Chem 1996;271:2935–40.
[32] Gesser B, Lund M, Lohse N, Vestergaard C, Matsushima K, Sindet-Pedersen S, et al. IL-8 induces T cell chemotaxis, suppresses IL-4, and up-regulates IL-8 production by $CD4^+$ T cells. J Leukoc Biol 1996;59:407–11.
[33] Morrissey PJ, Goodwin RG, Nordan RP, Anderson D, Grabstein KH, Cosman D, et al. Recombinant interleukin 7, pre-B cell growth factor, has costimulatory activity on purified mature T cells. J Exp Med 1989;169:707–16.
[34] van Roon JAG, Glaudemans CAFM, Bijlsma JWJ, Lafeber FPJG. Differentiation of naïve $CD4^+$ T cells towards T helper 2 cells is not impaired in rheumatoid arthritis patients. Arthritis Res Ther 2003; 5:R269–76.
[35] Giacalone B, D'Auria L, Bonifati C, Riccardi E, Mussi A, D'Agosto G, et al. Decreased interleukin-7 and transforming growth factor-beta1 levels in blister fluids as compared to the respective serum levels in patients with bullous pemphigoid. Opposite behavior of TNF-alpha, interleukin-4 and interleukin-10. Exp Dermatol 1998;7: 157–61.
[36] Ochi K, Kohriyama T, Higaki M, Ikeda J, Harada A, Nakamura S. Changes in serum macrophage-related factors in patients with chronic inflammatory demyelinating polyneuropathy caused by intravenous immunoglobulin therapy. J Neurol Sci 2003;208:43–50.
[37] Dalakas MC. Mechanisms of action of IVIg and therapeutic considerations in the treatment of acute and chronic demyelinating neuropathies. Neurology 2002;59:S13–21.
[38] Mathey EK, Pollard JD, Armati PJ. TNFα, IFNγ and IL-2 mRNA expression in CIDP sural nerve biopsies. J Neurol Sci 1999;163: 47–52.
[39] Misawa S, Kuwabara S, Mori M, Kawaguchi N, Yoshiyama Y, Hattori T. Serum levels of tumor necrosis factor-a in chronic inflammatory demyelinating polyneuropathy. Neurology 2001;56:666–9.
[40] Li Y-P, Pei Y-Y, Zhou Z-H, Zhang X-Y, Gu Z-H, Ding J, et al. PEGylated recombinant human tumor necrosis factor alpha:

pharmacokinetics and anti-tumor effects. Biol Pharm Bull 2001; 24:666–70.
[41] Horiuchi I, Ochi H, Murai H, Osoegawa M, Minohara M, Furuya H, et al. Th2 shift in mononeuritis multiplex and increase of Th2 cells in chronic inflammatory demyelinating polyneuropathy: an intracellular cytokine analysis. J Neurol Sci 2001;193:49–52.
[42] Raj DSC, Dominic EA, Wolfe R, Shah VO, Bankhurst A, Zager PG, et al. Coordinated increase in albumin, fibrinogen, and muscle protein synthesis during hemodialysis: role of cytokines. Am J Physiol Endocrinol Metab 2004;286:E658–64.
[43] Ertenli I, Kiraz S, Öztürk MA, Haznedaroğlu IC, Çelik İ, Çalgüneri M. Pathologic thrombopoiesis of rheumatoid arthritis. Rheumatol Int 2003;23:49–60.
[44] Anaya JM, Correa PA, Herrera M, Eskdale J, Gallagher G. Interleukin-10 (IL-10) influences autoimmune response in primary Sjögren's syndrome and is linked to IL-10 gene polymorphism. J Rheumatol 2002;29:1874–6.
[45] Zhou Y, Giscombe R, Huang D, Lefvert AK. Novel genetic association of Wegener's granulomatosis with the interleukin-10 gene. J Rheumatol 2002;29:317–20.
[46] Lard LR, van Gaalen FA, Schonkeren JJ, Pieterman EJ, Stoeken G, Vos K, et al. Association of the-2849 interleukin-10 promoter polymorphism with autoantibody production and joint destruction in rheumatoid arthritis. Arthritis Rheum 2003;48:1841–8.
[47] Li M-C, He S-H. IL-10 and its related cytokines for treatment of inflammatory bowel disease. World J Gastroenterol 2004;10:620–5.
[48] Willeke P, Schotte H, Erren M, Schlüter B, Mickholz E, Domschke W, et al. Concomitant reduction of disease activity and IL-10 secreting peripheral blood mononuclear cells during immunoadsorption in patients with active systemic lupus erythematosus. Cell Mol Biol 2002;48:323–9.
[49] Hattori N, Ichimura M, Nagamatsu M, Li M, Yamamoto K, Kumazawa K, et al. Clinicopathological features of Churg–Strauss syndrome-associated neuropathy. Brain 1999;122:427–39.

doi:10.1093/brain/awh453 *Brain* (2005), **128**, 988–1002

Intrathecal activation of the IL-17/IL-8 axis in opticospinal multiple sclerosis

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Summary

There are two distinct subtypes of multiple sclerosis in Asians, opticospinal (OS-multiple sclerosis) and conventional (C-multiple sclerosis). In OS-multiple sclerosis, selective and severe involvement of the optic nerves and spinal cord is characteristic, though its mechanisms are unknown. The present study aimed to find out possible differences in the cytokine/chemokine profiles in CSF between OS-multiple sclerosis and C-multiple sclerosis and to delineate the relationships between these profiles and neuroimaging and pathological features. Sixteen cytokines/chemokines, namely interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17, interferon (IFN)-γ, tumour necrosis factor (TNF)-α, granulocyte colony-stimulating factor (G-CSF), monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1β (MIP-1β), were measured simultaneously in CSF supernatants from 40 patients with relapsing–remitting multiple sclerosis (20 OS-multiple sclerosis and 20 C-multiple sclerosis) at relapse and 19 control patients with spinocerebellar degeneration (SCD), together with intracellular production of IFN-γ and IL-4 in CSF $CD4^+$ T cells. In CSF supernatants relative to controls, IL-17, MIP-1β, IL-1β and IL-13 were only significantly increased in OS-multiple sclerosis patients, while TNF-α was only significantly increased in C-multiple sclerosis patients, using a cut-off level of 1 pg/ml. IL-8 was significantly elevated in both OS-multiple sclerosis and C-multiple sclerosis patients. MCP-1 was significantly decreased in both OS-multiple sclerosis and C-multiple sclerosis patients, while IL-7 was only significantly decreased in C-multiple sclerosis patients. IL-17, IL-8 and IL-5 were significantly higher in OS-multiple sclerosis patients than in C-multiple sclerosis patients. The increases in IL-17 and IL-8 in OS-multiple sclerosis were still significant even after exclusion of the patients undergoing various immunomodulatory therapies. Assays of intracellular cytokine production revealed that both the $IFN\text{-}\gamma^+IL\text{-}4^-$ T-cell percentage and intracellular IFN-γ/IL-4 ratio in CSF cells were significantly greater in C-multiple sclerosis patients than in controls. Contrarily, OS-multiple sclerosis patients showed not only a significantly greater percentage of $IFN\text{-}\gamma^+IL\text{-}4^-$ T cells than controls but also a significantly higher percentage of $IFN\text{-}\gamma^-IL\text{-}4^+$ T cells than C-multiple sclerosis patients. Among the cytokines elevated in multiple sclerosis, only IL-8 showed a significant positive correlation with the Expanded Disability Status Scale of Kurtzke score. Both the length of the spinal cord lesions on MRI and the CSF/serum albumin ratio had a significant positive correlation with IL-8 and IL-17 in multiple sclerosis, in which the spinal cord lesions were significantly longer in OS-multiple sclerosis than in C-multiple sclerosis. Three of six spinal cord specimens from autopsied OS-multiple sclerosis cases demonstrated numerous myeloperoxidase-positive neutrophils infiltrating necrotic lesions. These findings strongly suggest that in OS-multiple sclerosis, in addition to the Th1 cell upregulation seen in C-multiple sclerosis, intrathecal activation of the IL-17/IL-8 axis inducing heavy neutrophil infiltration contributes to extensive spinal cord lesion formation.

Keywords: multiple sclerosis; cytokine; chemokine; cerebrospinal fluid; neutrophil

Abbreviations: C-multiple sclerosis = conventional form of multiple sclerosis; EAE = experimental allergic encephalomyelitis; EDSS = Expanded Disability Status Scale of Kurtzke; G-CSF = granulocyte colony-stimulating factor; IFN = interferon; IL = interleukin; LP = lumbar puncture; MCP-1 = monocyte chemoattractant protein-1; MIP-1β = macrophage

inflammatory protein-1β; NMO = neuromyelitis optica; OS-multiple sclerosis = opticospinal form of multiple sclerosis; PBL = peripheral blood lymphocyte; SCD = spinocerebellar degeneration; TNF = tumour necrosis factor

Received September 20, 2004. Revised October 24, 2004. Accepted January 26, 2005. Advance Access publication March 2, 2005

Introduction

Multiple sclerosis is a chronic inflammatory disease of the CNS characterized by macrophage and lymphocyte infiltration, demyelination, axonal injury and loss of neurological function. It has been hypothesized, but not yet proven, to be caused by an autoimmune mechanism targeting CNS myelin.

Cytokines are soluble proteins that mediate and regulate interactions between cells of the immune system, and are key mediators of autoimmune attack against CNS myelin (Huang *et al.*, 1999). In multiple sclerosis, many prior studies have documented that proinflammatory (Th1 type) cytokines such as interferon (IFN)-γ, tumour necrosis factor (TNF)-α, interleukin (IL)-1, IL-2 and IL-12 (p40) are involved in the onset and perpetuation of the disease, while, in contrast, anti-inflammatory (Th2 type) cytokines such as IL-4, IL-10 and transforming growth factor-β are downregulated during phases of disease activity and upregulated in phases of disease remission (Panitch *et al.*, 1987; Sharief and Hentges, 1991; Sharief and Thompson, 1993; Link *et al.*, 1994; Rieckmann *et al.*, 1994; Matusevicius *et al.*, 1996; Navikas and Link, 1996; Navikas *et al.*, 1996; Monteyne *et al.*, 1997; Fassbender *et al.*, 1998; Link, 1998; Huang *et al.*, 1999; van Boxel-Dezaire *et al.*, 1999).

It has been hypothesized that the progression of multiple sclerosis, and perhaps even its induction, may be causally related to dysregulation of the balance between Th1 and Th2 cytokines (Olsson, 1995). However, other reports have suggested that the Th1/Th2 cytokine paradigm for multiple sclerosis is an oversimplification, and that various other immune cells, including Th2, $CD8^+$ T and B cells, are involved in the complex and heterogeneous mechanisms in this condition (Laman *et al.*, 1998; Hemmer *et al.*, 2002; Lassmann and Ransohoff, 2004). Multiple cytokines often function as complexes, with the function of one inducing the function of another in a cascade effect. In this regard, multiplexed fluorescent bead-based immunoassays, which have a dynamic range of standard curves and require only small amounts of material for simultaneous measurements of numerous cytokines and chemokines, are more useful than enzyme-linked immunosorbent assay (ELISA) methods (Vignali, 2000; Kellar *et al.*, 2001; de Jager *et al.*, 2003).

Multiple sclerosis is rare in Asians but, when it does appear, the destruction of the optic nerves and spinal cord is striking (Kira, 2003). We previously reported the existence of two subtypes of multiple sclerosis in Japanese, the opticospinal form (OS-multiple sclerosis) that shows selective and severe involvement of the optic nerves and spinal cord, and the conventional form (C-multiple sclerosis) that shows disseminated lesions in the CNS including the cerebrum, cerebellum and brainstem (Kira *et al.*, 1996). The two subtypes have different clinical and neuroimaging features, and immunogenetic backgrounds (Kira *et al.*, 1996; Yamasaki *et al.*, 1999; Kira, 2003). OS-multiple sclerosis has distinct features, such as a higher age at onset, marked female preponderance and a higher Kurtzke's Expanded Disability Status Scale (EDSS) score (Kurtzke, 1983), resulting from severe visual impairment and marked spinal cord dysfunction compared with C-multiple sclerosis. Severe inflammatory destruction has been suggested in OS-multiple sclerosis, because of the occasionally higher cell counts and protein amounts in the CSF, as well as long swollen lesions extending over several vertebral segments on spinal cord MRI (Kira, 2003). Furthermore, pathological studies have revealed not only demyelination, but also axonal loss, necrosis, cavity formation, thickened vessel wall and capillary proliferation in OS-multiple sclerosis lesions (Shiraki, 1965; Ikuta *et al.*, 1982; Tabira and Tateishi, 1982). These clinico-pathological features of OS-multiple sclerosis in Asians are similar to those of a relapsing–remitting form of Devic's neuromyelitis optica (NMO) in a Western population (Wingerchuk *et al.*, 1999; Cree *et al.*, 2002; Lucchinetti *et al.*, 2002). Considerable overlap and common mechanisms between the two conditions are supposed, as seen in the recent discovery of NMO-IgG, commonly found in both (Lennon *et al.*, 2004); however, the immune mechanisms responsible for such distinct clinico-pathological features remain unknown.

Although many studies have been published on cytokine/chemokine alterations in multiple sclerosis, subtype-related alterations have been poorly characterized. We previously reported that OS-multiple sclerosis showed a significant Th1/Tc1 shift through relapse and remission phases in peripheral blood lymphocytes (PBLs), while C-multiple sclerosis only showed a significant Th1 shift during a relapse phase (Horiuchi *et al.*, 2000; Wu *et al.*, 2000; Ochi *et al.*, 2001). In CSF, except for one report disclosing a greater increase in macrophage migration inhibitory factor (MIF) in OS-multiple sclerosis than in C-multiple sclerosis (Niino *et al.*, 2000), OS-multiple sclerosis-related changes in CSF cytokine/chemokine profiles have not been investigated, probably because of the low concentrations of cytokines/chemokines and the fragility and limited numbers of CSF cells.

Therefore, in the present study, we attempted to uncover OS-multiple sclerosis-related cytokine/chemokine alterations in CSF that could explain the distinct neuroimaging and pathological features. First, we simultaneously measured 16 cytokines/chemokines, namely IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17, IFN-γ, TNF-α, granulocyte colony-stimulating factor (G-CSF),

Table 1 *Demographic features of OS-multiple sclerosis and C-multiple sclerosis patients*

	CSF supernatants assays		Intracellular cytokine production assays	
	OS-multiple sclerosis	C-multiple sclerosis	OS-multiple sclerosis	C-multiple sclerosis
No. of patients	20	20	11	13
Sex (F/M)	19/1	16/4	11/0	11/2
Age at onset (years)	44.2 ± 16.9*	25.8 ± 8.9	48.5 ± 21.8*	30.2 ± 15.2
Age at LP (years)	51.0 ± 15.1*	31.6 ± 9.6	54.6 ± 18.3*	37.2 ± 14.3
Duration of disease (years)	6.3 ± 5.4	6.1 ± 7.1	6.1 ± 5.0	7.2 ± 8.3
No. of relapses	7.3 ± 5.1	6.1 ± 5.7	5.7 ± 4.0	5.9 ± 6.0
EDSS	5.5 ± 2.3*	4.0 ± 1.8	5.0 ± 2.2	4.4 ± 1.8
CSF				
Cell count (/μl)	3.6 ± 8.0	4.2 ± 6.8	0.8 ± 1.2	1.8 ± 1.7
Total protein (mg/dl)	43.0 ± 22.5	37.7 ± 23.8	37.4 ± 15.2	39.6 ± 18.3
CSF/serum albumin ratio (10^{-4})	90.0 ± 44.0*	58.0 ± 41.2	91.5 ± 41.4*	52.0 ± 24.1
IgG index	0.674 ± 0.179	0.837 ± 0.414	0.843 ± 0.694	0.853 ± 0.306

Values are expressed as the mean ± SD. The CSF cell count and total protein amount in the control patients used for the CSF supernatant assays were 0.6 ± 0.6/μl and 28.6 ± 9.5 mg/dl, respectively, while those used for the intracellular cytokine production assays were 0.8 ± 1.1/μl and 33.5 ± 9.6 mg/dl, respectively. The numbers of patients whose CSF/serum albumin ratio and IgG index were measured were 14 in OS-multiple sclerosis and 16 in C-multiple sclerosis in the supernatants assays and eight in OS-multiple sclerosis and eight in C-multiple sclerosis in the intracellular cytokine production assays. OS-multiple sclerosis = opticospinal form of multiple sclerosis; C-multiple sclerosis = conventional form of multiple sclerosis; LP = lumbar puncture; EDSS = Expanded Disability Status Scale of Kurtzke. *$P < 0.05$.

monocyte chemoattractant protein 1 (MCP-1) and macrophage inflammatory protein 1β (MIP-1β), in the CSF from OS-multiple sclerosis and C-multiple sclerosis patients at relapse using a multiplexed fluorescent bead-based immunoassay. Secondly, we examined the intracellular production of IFN-γ and IL-4 in CSF $CD4^+$ T cells from OS-multiple sclerosis and C-multiple sclerosis patients by flow cytometry. Thirdly, we analysed relationships among CSF cytokine/chemokine changes and clinical and spinal cord MRI findings, as well as neuropathological findings for autopsied spinal cord specimens from multiple sclerosis patients.

Materials and methods

Patients

Cytokine and chemokine assays of CSF supernatants were performed using CSF from multiple sclerosis patients exclusively at the time of clinical relapse (within 30 days of the onset of acute or subacute exacerbation). For these assays, 40 patients with relapsing–remitting multiple sclerosis [five males and 35 females; age at examination: 41.5 ± 15.9 years (mean ± SD), range: 18–89] who were diagnosed with multiple sclerosis based on McDonald's diagnostic criteria (McDonald *et al.*, 2001) at the Department of Neurology, Kyushu University Hospital were enrolled in this study. In addition, 19 patients with spinocerebellar degeneration (SCD) (nine males and 10 females; age: 59.4 ± 10.9 years, range: 32–80) were used as controls. The multiple sclerosis patients were clinically classified into two subtypes: OS-multiple sclerosis and C-multiple sclerosis, as described previously (Kira *et al.*, 1996). Briefly, patients who showed a relapsing–remitting course and had both optic nerve and spinal cord involvement without any clinical evidence of disease in either the cerebrum or cerebellum were considered to have OS-multiple sclerosis. Patients who showed minor brainstem signs, such as transient double vision and nystagmus, in addition to opticospinal involvement, were included in this subtype. All other patients who showed multiple involvement of the CNS, including the cerebrum and cerebellum, were considered to have C-multiple sclerosis. The disability status of the patients was scored by one of the authors (T.I.) throughout the study, according to the EDSS (Kurtzke, 1983). The mean times from symptom onset to lumbar puncture (LP) were 13.9 days (range: 1–30 days) for OS-multiple sclerosis and 12.8 days (range: 1–30 days) for C-multiple sclerosis; the two did not differ significantly. Of the 40 multiple sclerosis patients, five C-multiple sclerosis and 10 OS-multiple sclerosis patients had received IFN-β or high-dose corticosteroids at the time of LP. The demographic features of the patients are summarized in Table 1. The ages at onset and LP, the EDSS score and CSF/serum albumin ratio were significantly higher in OS-multiple sclerosis than in C-multiple sclerosis ($P = 0.0000064$, 0.0000049, 0.019 and 0.023, respectively), while the disease duration, number of relapses, CSF cell count, total protein amount and IgG index did not differ significantly between the two.

For analysis of intracellular IFN-γ and IL-4 in CSF cells, 24 patients with multiple sclerosis (two males and 22 females; age: 45.2 ± 18.2 years, range: 21–89) who fulfilled McDonald's criteria and 12 control patients with various other non-inflammatory neurological diseases (seven males and five females; age: 52.4 ± 17.2 years, range: 20–75) were enrolled. In the multiple sclerosis group, 11 had OS-multiple sclerosis and 13 had C-multiple sclerosis. Of these, 21 were relapsing–remitting multiple sclerosis (11 OS-multiple sclerosis and 10 C-multiple sclerosis) and three were secondary progressive multiple sclerosis (three C-multiple sclerosis). The control group of 12 patients was comprised of two with amyotrophic lateral sclerosis, two with Alzheimer's disease, two with cervical spondylosis, one with SCD, one with Parkinson's disease, one with progressive supranuclear palsy, one with spinal cord infarction, one with epilepsy and one with conversion hysteria. The demographic features of the patients are also summarized in Table 1. The ages at onset and LP, and CSF/serum albumin ratio were significantly higher in OS-multiple sclerosis than in C-multiple sclerosis ($P = 0.017$, 0.028 and 0.028, respectively).

CSF sample collection

At least 5 ml of CSF was obtained from all patients by non-traumatic LP. Twenty-three CSF samples (all at the relapse phase) from 20 OS-multiple sclerosis patients, 22 CSF samples (all at the relapse phase) from 20 C-multiple sclerosis patients, and 19 CSF samples from 19 control patients were obtained for extracellular cytokine analysis. Neither multiple sclerosis nor control patients had any ongoing or recent infection at the time of LP. Intracellular IFN-γ and IL-4 analyses were performed using 11 CSF samples (10 at the relapse phase and one at the remission phase) from 11 OS-multiple sclerosis patients, 13 CSF samples (seven at the relapse phase, three at the remission phase and three at the progressive phase) from 13 C-multiple sclerosis patients, and 12 CSF samples from 12 control patients. CSF samples were immediately centrifuged at 800 r.p.m./min at 4°C for 5 min, and the supernatants stored at −70°C until analysis. The cell counts, total protein amounts, CSF/serum albumin ratio and IgG index used for the study are shown in Table 1.

Multiplexed fluorescent bead-based immunoassay

CSF supernatants were collected and analysed simultaneously for 16 different cytokines and chemokines, namely IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17, IFN-γ, TNF-α, G-CSF, MCP-1 and MIP-1β, using the Bio-Plex Cytokine Assay System (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions (Kellar *et al.*, 2001; de Jager *et al.*, 2003). Briefly, 50 μl of each CSF supernatant and various concentrations of each cytokine standard (Bio-Rad) were added to 50 μl of antibody-conjugated beads (Bio-Rad) in a 96-well filter plate (Millipore, Billerica, MA). After a 30 min incubation, the plate was washed and 25 μl of a biotinylated antibody solution (Bio-Rad) was added to each well, followed by another 30 min incubation. The plate was then washed and 50 μl of streptavidin-conjugated phycoerythrin (PE; Bio-Rad) was added to each well and incubated for 10 min. Following a final wash, the contents of each well were resuspended in 125 μl of assay buffer (Bio-Rad) and analysed using a Bio-Plex Array Reader (Bio-Rad). The cytokine concentrations were calculated by reference to a standard curve for each cytokine derived using various concentrations of the cytokine standards (0.2, 0.78, 3.13, 12.5, 50, 200, 800 and 3200 pg/ml) assayed in the same manner as the CSF samples. The same lots of monoclonal antibodies for Bio-Plex Cytokine Assay System were used throughout the experiments, and the inter- and intra-assay variability was reported to be <10% (manufacturer's instructions). The detection limit of each cytokine was determined by the recovery of the corresponding cytokine standard, and the lowest values showing >50% recovery were set as the lower detection limits. The lower detection limit for each cytokine was as follows: 0.2 pg/ml for IL-2, IL-4, IL-5, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17, IFN-γ and TNF-α, 0.78 pg/ml for IL-1β and IL-6, and 3.13 pg/ml for G-CSF, MCP-1 and MIP-1β. All samples were analysed undiluted in duplicate.

Intracellular cytokine analysis by flow cytometry

Each CSF supernatant was carefully removed and the cell sediment was suspended in RPMI 1640 (Nipro, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS; Gibco BRL, Gaithersburg, MD; Lot # 3217341S), followed by incubation with 25 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma, St Louis, MO), 1.0 μg/ml ionomycin (Sigma) and 10 μg/ml brefeldin A (BFA; Sigma) in a 24-well plate at 37°C for 4 h under 5% CO_2. After washing with phosphate-buffered saline containing 0.1% bovine serum albumin (0.1% BSA–PBS), cells were stained with perCP-conjugated anti-CD4 monoclonal antibodies (Immunotech, Marseille, France) and incubated on ice in the dark for 15 min. Following another wash with 0.1% BSA–PBS, fluorescence-activated cell sorting (FACS) permeabilizing solution (Becton Dickinson, San Jose, CA) was added and the cells were placed in the dark for 10 min. After two washes with 0.1% BSA–PBS, the cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-IFN-γ (Immunotech) and PE-conjugated anti-IL-4 (Immunotech) antibodies for intracellular cytokine analysis, or with mouse IgG2a–FITC (Immunotech) and IgG1–PE (Immunotech) as controls, respectively. After a 30 min incubation on ice in the dark, the percentages of intracellular IFN-γ- and IL-4-producing cells were immediately analysed by flow cytometry using an Epics XL System II (Coulter, Hialeah, FL). Analysis gates were first set on lymphocytes according to the forward and side scatter properties and then on $CD4^+$ lymphocytes. Cases with a $CD4^+$ cell count of <500 were discarded from the analysis to increase the reliability.

Neuroimaging of the spinal cord

For spinal cord MRI, T2-weighted (SE 2500–4900/113–116) and T1-weighted (SE 400–500/11–12) images were obtained in the sagittal and axial planes. For the contrast-enhanced study, MRI was initiated 2–3 min after intravenous administration of gadolinium-pentetic acid (0.1 mmol/kg), using the T1-weighted sequences in the sagittal and axial planes. Lengths of spinal cord lesions were expressed in cm. Longitudinally extensive spinal cord lesions were defined as those extending over three vertebral spine lengths, which are considered to be exceptional in multiple sclerosis (McDonald *et al.*, 2001). Spinal cord MRIs taken at the time of relapse when the CSF samples were drawn (within 30 days of the onset of acute or subacute exacerbation) were evaluated independently by two examiners, of whom one (F.M.) was an experienced neuroradiologist and was blinded to the diagnosis. Spinal cord MRIs at the relapse were available for 34 of 40 multiple sclerosis patients (17 OS-multiple sclerosis and 17 C-multiple sclerosis patients) and, of these, 30 had spinal cord symptomatology (17 OS-multiple sclerosis and 13 C-multiple sclerosis).

Histopathological analysis of infiltrating cells in autopsied spinal cord specimens of OS-multiple sclerosis and C-multiple sclerosis

For the neuropathological analysis, spinal cord specimens obtained at autopsy from six OS-multiple sclerosis and two C-multiple sclerosis cases were used. Each autopsied specimen was fixed in 10% buffered formalin for several weeks, and then embedded in paraffin. Sections were either stained with haematoxylin and eosin, or immunostained using rabbit polyclonal antibodies against myeloperoxidase (1 : 500; NeoMarkers, Fremont, CA) or myelin basic protein (1 : 200; DAKO, Denmark) or mouse monoclonal antibodies against phosphorylated neurofilaments (1 : 200; DAKO; clone 2F11), macrophages (1 : 500; DAKO; clone KP1), T-cell antigen (1 : 500; DAKO; clone UCHL1) or B-cell antigen (1 : 500; DAKO; clone L26). Autoclave or microwave pre-treatment was performed to retrieve the antigens for immunostaining. The number of infiltrating cells, including neutrophils, macrophages, and T and B cells, was averaged for four separate fields (each field ×200) in the lesion in the same cross-section of the spinal cord.

Statistical analysis

Fisher's exact probability test was employed for comparisons of the detection rates of cytokines and chemokines in each group. The non-parametric Mann-Whitney *U* test was employed for comparison of the cytokine and chemokine levels in each group. The difference between PBLs and CSF in each group was analysed by Wilcoxon signed-rank test. Statistical significance was set at $P < 0.05$. Spearman's rank correlation analysis was used for statistical analysis of correlations between various clinical parameters and CSF cytokine levels.

Results

Detection rates of each cytokine and chemokine in CSF supernatants

The detection rate of IL-10 was significantly higher in OS-multiple sclerosis and C-multiple sclerosis patients than in control patients (91.3 versus 36.8%, $P = 0.00027$ in OS-multiple sclerosis; 81.8 versus 36.8%, $P = 0.0046$ in C-multiple sclerosis), while that of IL-7 was significantly lower in OS-multiple sclerosis and C-multiple sclerosis patients than in control patients (60.9 versus 100%, $P = 0.002$ in OS-multiple sclerosis; 68.2 versus 100%, $P = 0.0098$ in C-multiple sclerosis). In addition, IL-17 was significantly higher in OS-multiple sclerosis patients than in control patients (73.9 versus 36.8%, $P = 0.028$), while TNF-α was significantly higher in C-multiple sclerosis patients than in control patients (77.3 versus 42.1%, $P = 0.029$). The detection rates of other cytokines did not differ significantly between control patients and the two multiple sclerosis subtypes. When the five C-multiple sclerosis and 10 OS-multiple sclerosis patients on immunomodulatory therapies were excluded, essentially the same results were obtained, except that the increased detection rate of TNF-α in C-multiple sclerosis lost significance. IL-2 was not used for further statistical analysis because of its extremely low detection rate (<20% in total) in CSF.

Comparison among diseases of cytokine and chemokine levels in CSF supernatants

The cytokine and chemokine levels in CSF supernatants from controls, OS-multiple sclerosis and C-multiple sclerosis patients analysed by the multiplexed fluorescent bead-based immunoassay are shown in Fig. 1. Among the 16 cytokines examined, IL-17, MIP-1β, IL-13 and IL-1β were only significantly increased in OS-multiple sclerosis patients compared with control patients. IL-8, IL-10 and TNF-α were significantly increased in both OS-multiple sclerosis and C-multiple sclerosis patients compared with control patients. On the other hand, IL-7 and MCP-1 were significantly decreased in both OS-multiple sclerosis and C-multiple sclerosis patients compared with control patients. When the cytokine levels were compared between the two multiple sclerosis subtypes, IL-17, IL-8 and IL-5 were significantly increased in OS-multiple sclerosis patients compared with C-multiple sclerosis patients.

Considering the possibly low reliability for the lower ranges of the cytokine/chemokine concentrations (<1 pg/ml), we set 1 pg/ml as the cut-off level and reanalysed the data. Even when this cut-off level was used, the increased IL-17, MIP-1β, IL-13 and IL-1β in OS-multiple sclerosis patients, increased TNF-α in C-multiple sclerosis patients, increased IL-8 in both OS-multiple sclerosis and C-multiple sclerosis patients, decreased IL-7 in C-multiple sclerosis patients and decreased MCP-1 in both OS-multiple sclerosis and C-multiple sclerosis patients compared with control patients, and higher levels of IL-17, IL-8 and IL-5 in OS-multiple sclerosis patients than in C-multiple sclerosis patients were all still statistically significant. When the five C-multiple sclerosis and 10 OS-multiple sclerosis patients on immunomodulatory therapies were excluded, the increased IL-17 and MIP-1β in OS-multiple sclerosis patients, increased IL-8 in both OS-multiple sclerosis and C-multiple sclerosis patients, and decreased MCP-1 in C-multiple sclerosis patients compared with control patients, and higher levels of IL-17 in OS-multiple sclerosis patients than in C-multiple sclerosis patients still held statistical significance using this cut-off level. The statistical significances for the cytokine/chemokine changes are summarized in Table 2 according to cut-off level and the presence or absence of immunomodulatory therapies.

Comparison of the intracellular cytokine production in CD4$^+$ T cells between CSF and PBLs and among diseases

IFN-γ$^+$IL-4$^-$ CD4$^+$ T-cell percentages were significantly higher in CSF cells than in PBLs for all three groups (Fig. 2). In PBLs, there were no significant differences in the IFN-γ$^+$IL-4$^-$ CD4$^+$ T-cell percentages among the three groups, while in CSF cells, IFN-γ$^+$IL-4$^-$ CD4$^+$ T-cell percentages were significantly increased in both OS-multiple sclerosis and C-multiple sclerosis patients compared with control patients. For the IFN-γ$^-$IL-4$^+$ CD4$^+$ T-cell percentages, CSF cells only showed a significantly lower value than PBLs in control patients. There was no significant change in the IFN-γ$^-$IL-4$^+$ T-cell percentages between PBLs and CSF cells in either OS-multiple sclerosis or C-multiple sclerosis patients, and in OS-multiple sclerosis patients, CSF cells even showed a higher IFN-γ$^-$IL-4$^+$ T-cell percentage than PBLs. In PBLs, IFN-γ$^-$IL-4$^+$ CD4$^+$ T-cell percentages were significantly decreased in OS-multiple sclerosis and C-multiple sclerosis patients compared with control patients. In CSF cells, IFN-γ$^-$IL-4$^+$ CD4$^+$ T-cell percentages did not differ significantly between control and OS-multiple sclerosis patients, while C-multiple sclerosis patients had a significantly lower IFN-γ$^-$IL-4$^+$ CD4$^+$ T-cell percentage than OS-multiple sclerosis patients, and showed a lower value than control patients although the difference was not significant.

Consequently, the intracellular IFN-γ/IL-4 ratio in CD4$^+$ T cells was significantly higher in CSF cells than in PBLs in