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CSF cytokine and chemokine profiles in acute disseminated encephalomyelitis

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Abstract

We simultaneously measured 16 cytokines/chemokines in cerebrospinal fluid (CSF) from 14 patients with acute disseminated encephalomyelitis (ADEM) and 20 controls using a fluorescent bead-based immunoassay. A variety of cytokines, such as IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFN-γ, TNF-α, G-CSF and MIP-1β, were significantly elevated in ADEM. In particular, G-CSF showed a marked 38-fold increase compared to the control mean. Significant positive correlations with inflammatory parameters in CSF, such as cell counts and protein levels, were found for IFN-γ, IL-6 and IL-8. In contrast, IL-17 produced by activated $CD4^+$ memory T cells was not increased. The results suggested that various cytokines related to activation of macrophages/microglias and Th_1 and Th_2 cells are upregulated in CSF in ADEM.

Keywords: Acute disseminated encephalomyelitis; Cytokine; Chemokine; Cerebrospinal fluid

1. Introduction

Acute disseminated encephalomyelitis (ADEM) is an acute inflammatory demyelinating disease of the central nervous system (CNS) that preferentially affects children and young adults. It typically occurs after infections or vaccination (Dale et al., 2000) and is therefore believed to result from a transient autoimmune response against myelin, possibly via molecular mimicry or non-specific activation of autoreactive T cells (Garg, 2003). Cytokines and chemokines are key mediators of autoimmune diseases and may also play roles in the evolution of ADEM (Dale, 2003; Garg, 2003). However, just a few studies have measured limited numbers of cytokines in cerebrospinal fluid (CSF) from ADEM patients using enzyme-linked immunosorbent assays (ELISAs), and alterations were only found in interleukin (IL)-6 (Dale and Morovat, 2003; Ichiyama et al., 2002) and IL-10 (Ichiyama et al., 2002). A recently developed multiplexed fluorescent bead-based immunoassay is able to simultaneously measure multiple cytokines and chemokines using only small volumes of materials, and is therefore especially suitable for assaying cytokines in CSF. Using this method, we have successfully characterized disease-specific changes in CSF cytokines and chemokines in multiple sclerosis (MS) (Ishizu et al., 2005) and chronic inflammatory demyelinating polyneuropathy (CIDP) (Mei et al., 2005). In the present study we applied this method to simultaneously measure 16 cytokines/chemokines in ADEM CSF and clarify their possible roles in this condition.

2. Patients and methods

2.1. Patients

We performed cytokine and chemokine assays using CSF from 14 patients with ADEM (Table 1). The diagnosis of

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Table 1
Demographic features of the ADEM and OND patients

	ADEM	OND
Number of patients	14	20
Gender (male/female)	6/8	6/14
Age (years)	6.5±4.8	4.1±3.9
CSF		
Cell count (per μl)	96.1±120.9*	1.8±2.5
Total protein (mg/dl)	59.4±31.1*	22.8±11.7

Values are expressed as the mean±S.D. CSF data on cell counts and total protein amounts were not available in 2 ADEM patients.
ADEM=acute disseminated encephalomyelitis; OND=other non-inflammatory neurological diseases; CSF=cerebrospinal fluid.
* $p<0.001$ compared with OND.

ADEM was made based on MRI findings, consistent with a disseminated demyelinating process, as described previously in the Departments of Pediatrics in Yamaguchi and Kyushu University Hospitals (Ichiyama et al., 2002). Precipitating infections, such as upper respiratory tract infection, gastroenteritis and mumps, were observed in 7 of the 14 patients and a preceding fever in another 3. All 12 patients whose CSF data were available had either pleocytosis (≥5/μl) or total protein increase (≥40 mg/dl); mononuclear pleocytosis was seen in 11 of the 12 (92%) and protein increase in 8 (67%). All but one, who had residual mild hemiparesis, of the 14 patients showed complete recovery without any recurrence. The mean time from symptom onset to lumbar puncture (LP) was 8.5 days and patients who had received immunomodulatory therapies were excluded. In addition, 20 patients of similar ages with other non-inflammatory neurological diseases (OND) were used as controls (Table 1). The OND group consisted of 11 patients with epilepsy, 2 with cerebral palsy, and 1 each with congenital myopathy, narcolepsy, Leigh encephalopathy, Krabbe disease, holoprosencephaly, cortical dysplasia, and malingering. CSF was obtained from all patients by non-traumatic LP for diagnostic purposes and stored at −70 °C until analysis.

2.2. Multiplexed fluorescent bead-based immunoassay

CSF was 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, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, granulocyte colony-stimulating factor (G-CSF), monocyte chemoattractant protein 1 (MCP-1) and macrophage inflammatory protein 1β (MIP-1β), using the Bio-Plex Cytokine Assay System (Bio-Rad Laboratories, Hercules, CA) as described previously (Ishizu et al., 2005; Mei et al., 2005). Briefly, 50 μl of each CSF and various concentrations of each cytokine standard were added to 50 μl of antibody-conjugated beads in a 96-well filter plate. After incubation for 30 min, the plate was washed and 25 μl of a biotinylated antibody solution was added to each well, followed by incubation for another 30 min. The plate was then washed and 50 μl of streptavidin-conjugated phycoerythrin was added to each well and incubated for 10 min. Following a final wash, the contents of each well were resuspended in 125 μl assay buffer and analyzed using a Bio-Plex Array Reader (Bio-Rad). Cytokine concentrations were calculated by comparison with a standard curve for each cytokine derived from 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. All 34 CSF samples were measured at one time; intra-assay variability, expressed as a coefficient of variation, is reported to be less than 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 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 (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 analyzed undiluted in duplicate. Intra-assay variability between the duplicate determinations was calculated and expressed with %CV as follows: IL-1β=3.8±2.7%, IL-2=4.6±3.7%, IL-4=2.9±2.7%, IL-5=4.5±3.5%, IL-6=6.9±8.7%, IL-7=3.6±2.5%, IL-8=3.1±4.6%, IL-10=4.9±3.0%, IL-12 (p70)=4.1±3.0%, IL-13=3.3±2.7%, IL-17=3.6±2.4%, IFN-γ=4.5±4.2%, TNF-α=5.1±4.1%, G-CSF=4.7±3.6%, MCP-1=5.3±4.7%, MIP-1β=3.6±5.7%.

2.3. Statistical analysis

We used the following statistical tests for the appropriate applications: Fisher's exact probability test was used to compare the detection rates of cytokines and chemokines in each group; the non-parametric Mann–Whitney *U*-test was employed to compare the cytokine and chemokine levels in each group; and Spearman's rank correlation analysis was used to correlate various clinical parameters and CSF cytokine levels. Statistical significance was set at $p<0.05$.

3. Results

3.1. Comparisons of cytokine and chemokine detection rates in CSF

The detection rates of IL-1β, IL-2, IL-4, IL-5, IL-10, IFN-γ, TNF-α and G-CSF were significantly higher in ADEM than OND patients (100% vs. 5%, $p<0.0001$ for IL-1β; 71.4% vs. 15%, $p=0.001$ for IL-2; 100% vs. 65%, $p=0.02$ for IL-4; 100% vs. 50%, $p=0.001$ for IL-5; 100% vs. 45%, $p=0.0006$ for IL-10; 100% vs. 55%, $p=0.004$ for IFN-γ; 100% vs. 45%, $p=0.0006$ for TNF-α; 100% vs. 60%, $p=0.01$ for G-CSF). The detection rates of the other

cytokines did not differ significantly between ADEM and OND patients.

3.2. Comparisons of cytokine and chemokine levels in CSF

The following cytokines were significantly higher in ADEM than OND patients (Fig. 1): IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFN-γ, TNF-α, G-CSF and MIP-1β. The other cytokines (IL-7, IL-12 (p70), IL-13, IL-17 and MCP-1) did not differ significantly between the two groups. Even when the cut-off line was uniformly set at 2 pg/ml, the following cytokines were significantly higher in ADEM than OND patients; IL-1β, IL-2, IL-5, IL-6, IL-8, IL-10, TNF-α, G-CSF and MIP-1β, while IL-4 and IFN-γ lost significance. Nine of the 20 OND patients showed relatively high IFN-γ levels (more than 5 pg/ml). The diagnoses of these patients were; epilepsy in 6, and cortical dysplasia, Krabbe disease and Leigh encephalopathy in 1 each. Thus, 6 of the 11 patients with epilepsy (54.5%) making up part of the non-inflammatory neurological controls had unexpectedly high IFN-γ levels.

3.3. Correlations between clinical parameters and CSF cytokine levels in ADEM

Among the elevated cytokines/chemokines in ADEM CSF, IL-6 and IFN-γ showed significant positive correlations with the CSF cell count, while IL-8 showed a significant positive correlation with the CSF protein concentration (Fig. 2). The age at onset, gender and days from symptom onset to LP did not show any significant correlations with CSF cytokine/chemokine levels.

4. Discussion

The present study is the first to reveal that a variety of cytokines related to the activation of macrophages and Th_1 and Th_2 cells were significantly elevated in ADEM CSF, while a cytokine produced by activated $CD4^+$ memory T cells (IL-17) was not. Even when the cut-off line was set at 2 pg/ml, most of these cytokines remained statistically significance; however, the validity of such low cytokine

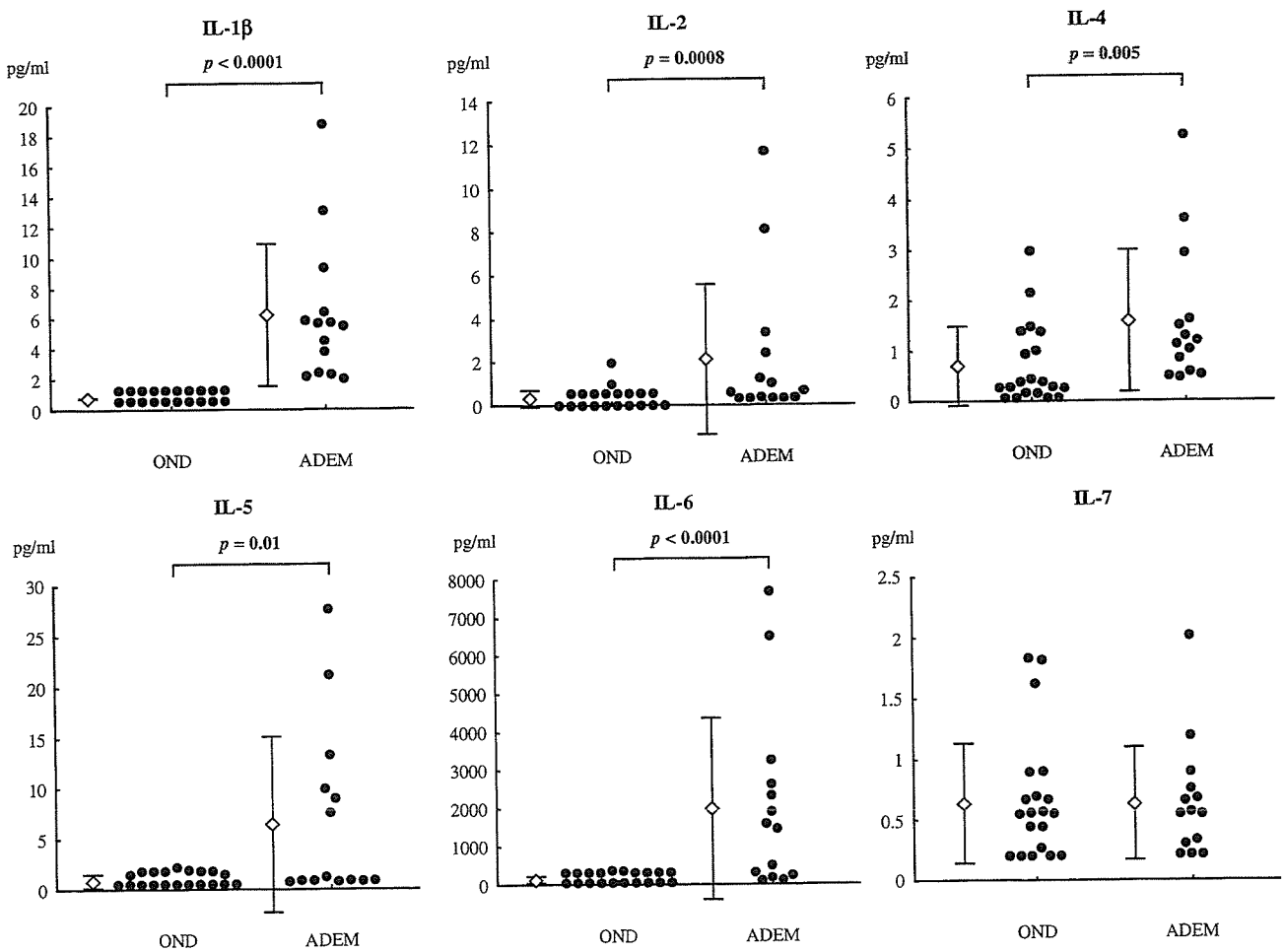

Fig. 1. Cytokine and chemokine levels in CSF from patients with ADEM and OND assessed by a multiplexed fluorescent bead-based immunoassay. Samples totaled 34: 14 ADEM and 20 OND. Open diamonds and bars indicate the mean±S.D. for each group.

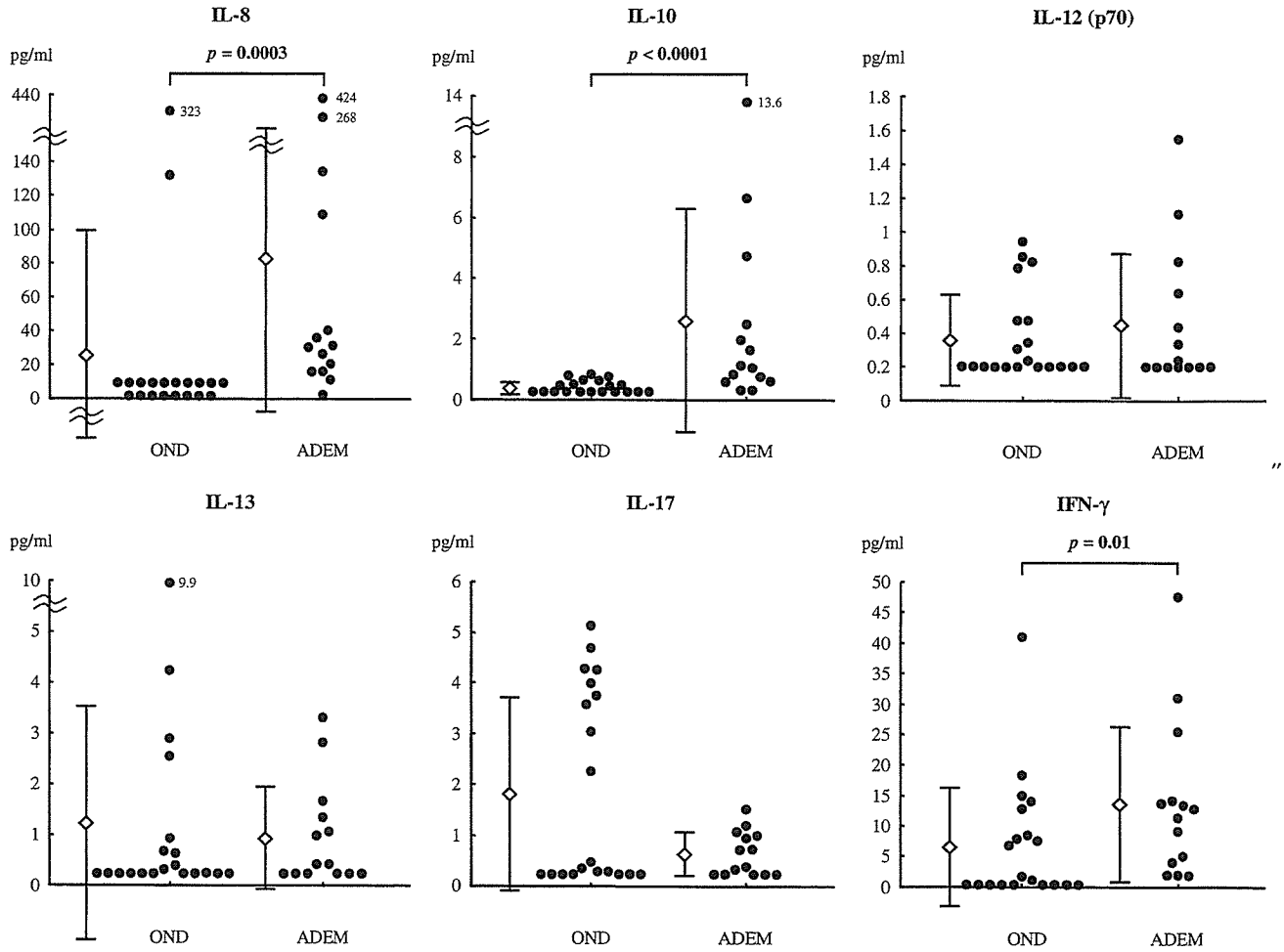

Fig. 1 (*continued*).

levels, less than 2 pg/ml, needs further study on a larger scale.

Involvement of type 1 cytokines in ADEM has been extremely difficult to demonstrate by ordinary ELISAs for CSF, with just one previous study reporting an increase in just TNF-α in a fraction of ADEM patients (17%), primarily due to the short half-lives of these Th_1 cytokines (Ichiyama et al., 2002). However, we have successfully demonstrated upregulation of Th_1 cytokines and found positive correlations between CSF inflammatory parameters and Th_1 (IFN-γ) and downstream inflammatory (IL-6 and IL-8) cytokines, further underscoring the contribution of Th_1 cells to inflammation of the CNS in ADEM. Th_1 cells have been shown to be deeply involved in organ-specific autoimmune diseases and are well known to induce experimental autoimmune encephalomyelitis (EAE), an animal model of acute inflammatory demyelinating diseases in humans. Taking all these observations into account, although the mechanism remains to be elucidated, Th_1 cells also appear to be involved in the inflammation of the CNS in ADEM.

In this study, we also noted a marked increase in G-CSF (38-fold) that has not been observed in other autoimmune inflammatory demyelinating diseases such as MS (Ishizu et al., 2005) and CIDP (Mei et al., 2005). G-CSF is mainly produced by monocytes/macrophages, endothelial cells, fibroblasts and mesothelial cells. G-CSF is not only a potent inducer of granulocytes, but also an inducer of type 2 cytokines, such as IL-4, IL-5 and IL-6, and a strong suppressor of EAE (Zavala et al., 2002), a Th_1-mediated autoimmune disease against CNS myelin. These facts suggest that G-CSF behaves like a Th_2 cytokine. Therefore, together with other Th_2 cytokines, G-CSF may be upregulated due to the host's effort to overcome Th_1-mediated inflammation. Alternatively, since anti-myelin reactive T cells isolated from peripheral blood of ADEM patients show a Th_2-biased profile (Jorens et al., 2000), and CNS myelin-reactive Th_2 cells by themselves can induce EAE (Lafaille et al., 1997), upregulated Th_2 cytokines may contribute to the exacerbation of ADEM, by working with the markedly increased amount of IL-6 and IL-10, through enhancement of

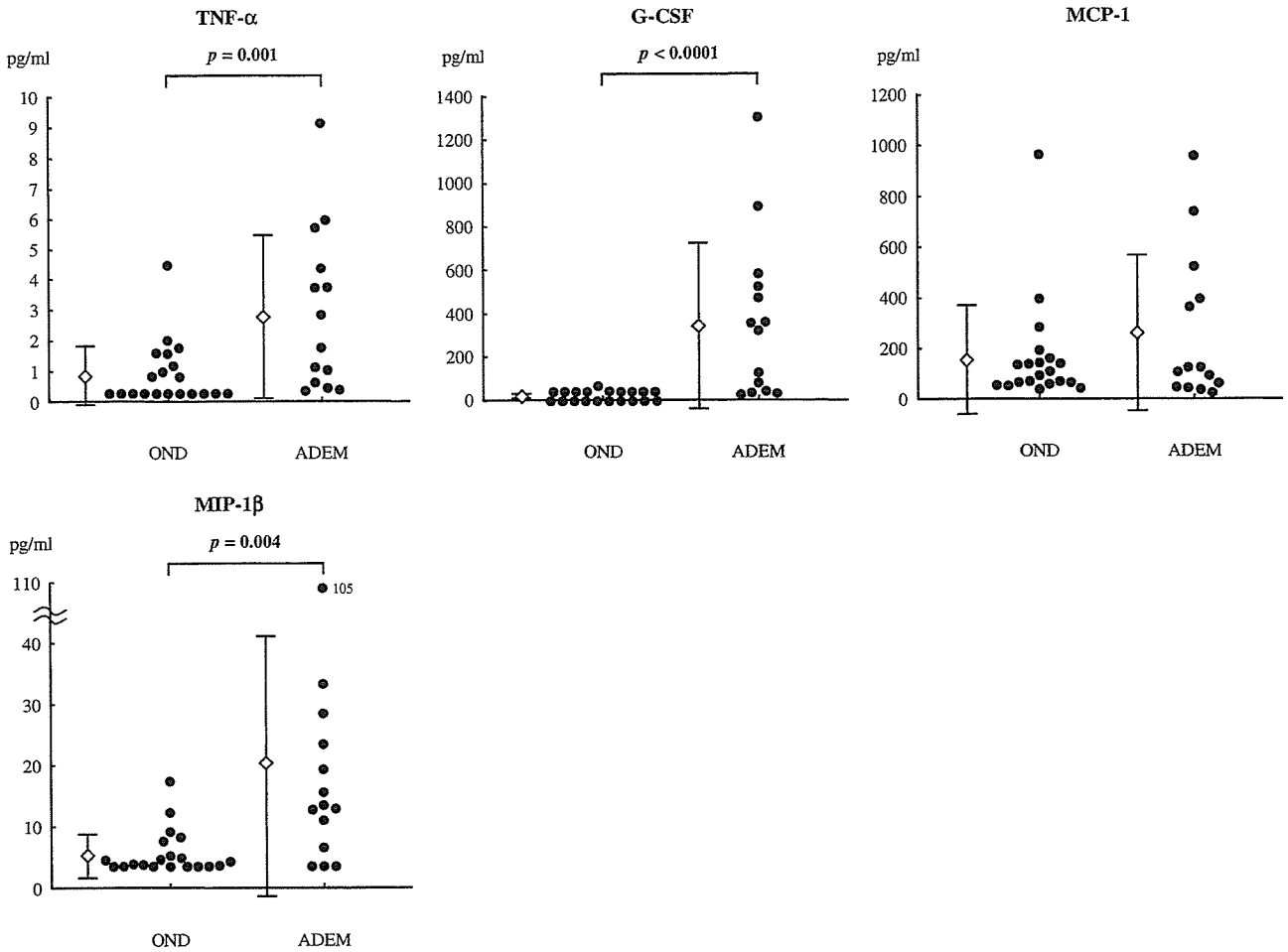

Fig. 1 (*continued*).

humoral immunity involving autoantibodies, complements and immune complexes (Dale et al., 2001; Jorens et al., 2000).

In addition, cytokines reflecting activation of macrophages/microglias, such as IL-1β, IL-6 and MIP-1β, as well as those acting on other effectors, such as IL-5 on eosinophils and IL-8 and G-CSF on granulocytes, were markedly elevated in CSF, indicating contribution of these cells to ADEM pathology. This may explain the infiltration of various types of inflammatory cells into ADEM lesions (Dale, 2003; Dale et al., 2000). However, it is intriguing to note that the marked upregulation of IL-17 that is observed in persistent autoimmune diseases against myelin such as MS (Ishizu et al., 2005) and CIDP (Mei et al., 2005), was not evident in ADEM. Activated $CD4^+$ memory T cells induced by IL-23 to mediate autoimmune inflammation mainly produce IL-17 (Kolls and Linden, 2004). It has been shown that IL-23, but not IL-12, is required for CNS autoimmunity (Cua et al., 2003; Langrish et al., 2005). IL-23 promotes the development of IL-17-producing $CD4^+$ T cells, whereas IL-12 drives IFN-γ-producing $CD4^+$ T cells (Langrish et al., 2005, 2004). The former are considered essential for organ-specific autoimmune inflammation, while the latter are critical for host defense against pathogens (Langrish et al., 2005, 2004; Shtrichman and Samuel, 2001). According to the results of our study, the latter process appears to be activated in ADEM, but not the former, which makes sense since ADEM usually follows infection by pathogens, as also seen in our series. We recently found that IL-17 was significantly higher in MS CSF at relapse than in non-inflammatory controls, while IFN-γ was not (Ishizu et al., 2005). Contrarily, the present study revealed that IFN-γ was increased in ADEM, while IL-17 was not. Therefore, these two conditions have distinct CSF cytokine profiles, suggesting that distinct immune mechanisms are operating. IL-17 may be involved in a persistent autoimmune attack against myelin, but not in a transient autoimmune attack such as ADEM, or at least in the first attack against myelin since the possibility that our ADEM patients may later relapse cannot be completely ruled out.

To summarize, this study has revealed a distinct CSF cytokine/chemokine profile for ADEM that may be useful for differentiating this disease from persistent autoimmune

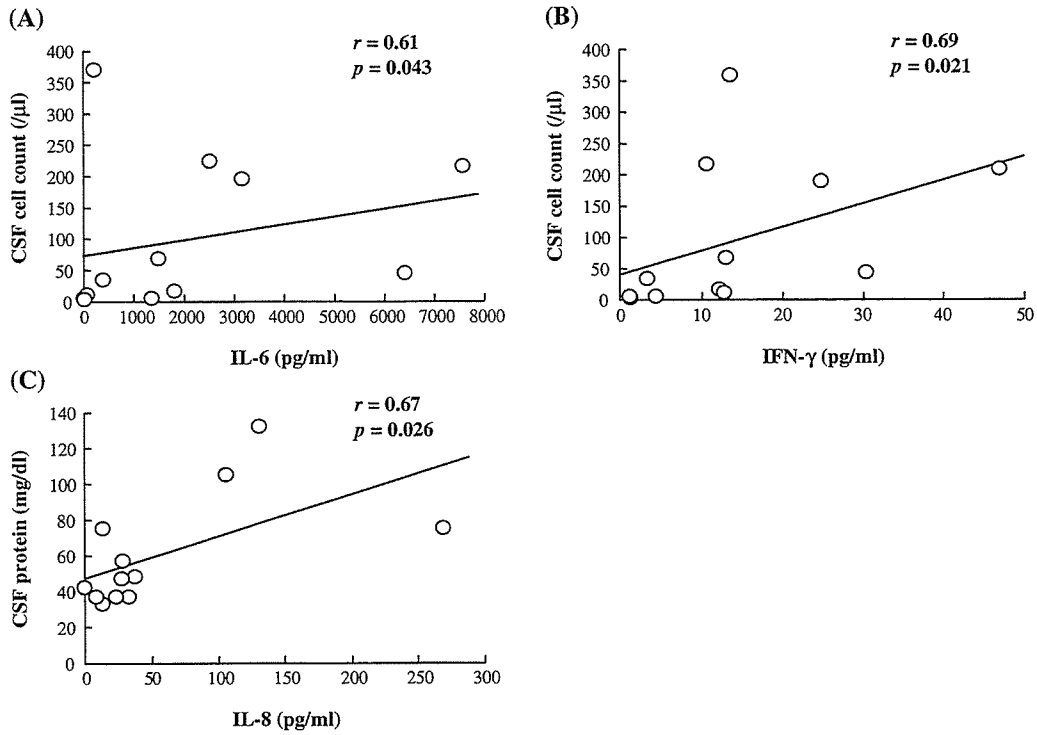

Fig. 2. Correlations between CSF parameters and cytokine/chemokine levels in patients with ADEM evaluated by Spearman's rank correlation analysis. (A) CSF cell count and IL-6 levels. (B) CSF cell count and IFN-γ levels. (C) CSF protein concentration and IL-8 levels.

demyelinating diseases, such as MS. Although we found no significant correlation between any of the cytokines in CSF and the days from symptom onset to LP, future larger-scale studies using CSF taken at multiple time points may clarify the relationship between cytokines and disease course.

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Upregulation of myeloperoxidase in patients with opticospinal multiple sclerosis: Positive correlation with disease severity

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Abstract

To clarify the role of myeloperoxidase (MPO) in multiple sclerosis (MS), we measured serum MPO levels in 86 Japanese patients with relapsing remitting MS, 47 with opticospinal MS (OSMS) and 39 with conventional MS (CMS), and 85 healthy subjects by sandwich enzyme immunoassays and analyzed relationships with clinical features. We found a significant increase in serum MPO in OSMS patients at relapse and remission, and in CMS patients at remission compared with controls. By logistic regression analysis, the clinical variable associated with high level of MPO at remission in OSMS patients (higher than the mean±2 S.D. of healthy controls) was only Kurtzke's Expanded Disability Status Scale (EDSS) score in blood sampling (p=0.0245); that is, a greater EDSS scores in the high MPO group, whereas in CMS none were associated. The results of our study suggest that MPO levels in remission are related with severe tissue destruction in OSMS.

Keywords: Multiple sclerosis; Myeloperoxidase; Opticospinal MS; Longitudinally extensive spinal cord lesion

1. Introduction

Multiple sclerosis (MS) is a human inflammatory demyelinating disease of the central nervous system (CNS) of unknown etiology. Although the mechanism of demyelination remains to be elucidated, the involvement of oxidative stress has been postulated (Hunter et al., 1984; Korpela et al., 1989). Myeloperoxidase (MPO) catalyzes the formation of hypochlorous acid, a very strong oxidant, and is expressed in granulocytes and macrophages/monocytes and in high concentrations in the primary granules. In some reports, genetic polymorphism of the gene associated with high producers, which has a G allele at position −463 in the promoter region, is associated with MS, especially early onset MS in females (Nagra et al., 1997), and severe tissue destruction (Zakrzewska-Pniewska et al., 2004); however, in other reports this is not the case (Kantarci et al., 2000; Nelissen et al., 2000). For example, one recent study showed a decrease in leukocyte MPO activity in benign MS and secondary and primary progressive MS in Caucasians (Ramsaransing et al., 2003). However, apart from this report, serum MPO levels in MS have not been reported in either Caucasians or non-Caucasians and thus the involvement of MPO in MS remains controversial.

In Asians, there are two distinct forms of MS: conventional (CMS) and opticospinal (OSMS) (Kira, 2003). Recently, we reported intrathecal upregulation of the IL-17/IL-8 axis in Japanese MS patients, especially those with OSMS (Ishizu et al., 2005); IL-8 is known to strongly activate granulocytes. MPO-positive granulocytes are also abundant in severely disrupted spinal cord tissues of OSMS patients (Ishizu et al., 2005). Therefore, in this study, we measured serum MPO levels in Japanese patients with relapsing remitting MS and analyzed relationships with clinical features in order to clarify the roles of MPO in MS.

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2. Subjects and methods

2.1. Subjects

A total of 86 consecutive patients (17 men and 69 women) with relapsing remitting MS, diagnosed according to the criteria of McDonald et al. (2001) at the Department of Neurology, Kyushu University Hospital, were enrolled in the present study after informed consent was obtained. None were receiving immunomodulatory therapies such as interferon beta, immunosuppressants or more than 20 mg of prednisolone per day at the time of blood sampling. The mean age at examination was 45.3 ±12.8 years (range=21 to 73 years) and the age at disease onset was 33.0±14.3 years (range=9 to 70 years). Anti-neutrophil cytoplasmic antibodies were negative in all cases. Patients were clinically classified as either OSMS or CMS before sandwich enzyme immunoassay of MPO was performed, as described previously (Kira et al., 1996). Briefly, 47 patients whose clinically estimated main lesions were confined to the optic nerves and spinal cord were classified as OSMS. These patients had no clinical evidence of disease in either the cerebrum or cerebellum, but minor brainstem signs, such as transient double vision and nystagmus, were acceptable. The remaining 39 patients had multiple involvement of the CNS, including the cerebrum, cerebellum and brainstem, and were classified as CMS. Disability was evaluated throughout by one of the authors (TM) using Kurtzke's Expanded Disability Status Scale (EDSS) score (Kurtzke, 1983). The demographic features of the patients are summarized in Table 1. Sera were obtained at relapse (within 1 month after onset of acute relapse) or remission. In the OSMS group, 15 serum samples were obtained at relapse and 40 at remission (8 patients were examined both at relapse and remission), while in the CMS group 13 serum samples were obtained at relapse and 32 at remission (6 patients were examined both at relapse and remission). A total of 24 MS samples were taken at the time when patients were on low-dose oral prednisolone (20 mg or less than 20 mg/day); 12 OSMS samples in remission, 4 OSMS samples in relapse, 6 CMS samples in remission, 2 CMS samples in relapse. Eighty-five healthy subjects (21 men and 64 women) were enrolled as control subjects. The average age at sampling was 43.5 ±12.6 years (range=21 to 64 years). Age at blood sampling was not significantly different between MS patients and controls.

2.2. Sandwich enzyme immunoassays

MPO was measured with quantitative sandwich enzyme immunoassays according to the manufacturer's standard protocol (Immundiagnostik AG, Bensheim, Germany). All measurements were conducted by one of the authors (WL) who was unaware of the diagnoses. Serum samples were thawed from −80 °C to room temperature and assayed in duplicate in 96-well polystyrene microtiter plates coated with a capture antibody. Peroxidase-conjugated polyclonal anti-human MPO antibody was used in the assays, and tetramethylbenzidine was used as the peroxidase substrate. The lower detection limit was 1.6 ng/ml.

2.3. Magnetic resonance imaging of the spinal cord

Magnetic resonance imaging (MRI) studies of the spinal cord were performed using a 1.5-T imager Magnetom Vision and Symphony (Siemens Medical Systems, Erlangen, Germany). The typical imaging parameters were as follows: sagittal T2-weighted turbo spin-echo imaging using the TR/TE range=2500–2800/90–116 ms, flip angle=180°, number of excitations=3–4; sagittal T1-weighted spin-echo imaging using the TR/TE range=400–440/11–12 ms, flip angle range=90°–170°, number of excitations=2–3; axial T2-weighted turbo spin-echo imaging using the TR/TE range=3200–5360/99–116 ms, flip angle=180°, number of excitations=3–4; and axial T1-weighted spin-echo imaging using the TR/TE range=400–440/12 ms, flip angle range=90°–170°, number of excitations=2. For sagittal imaging, a matrix of 256×56 or 512×12, a slice thickness of 4 mm and a slice gap of 0.4 mm were used, and for axial imaging, a matrix of 256×56 or 512×12, a slice thickness of 5 mm, and a slice gap range of 1.5–5 mm were used. Spinal cord lesions longer than three vertebral lengths were considered to be longitudinally extensive spinal cord lesions (LESCL).

Table 1
Demographic features of the subjects

	OSMS		CMS		Healthy controls
	Remission	Relapse	Remission	Relapse	
Number	40	15	32	13	85
Male/Female	5 : 35	2 : 13	9 : 23	4 : 9	21 : 64
	(1 : 7.00)	(1 : 6.50)	(1 : 2.17)	(1 : 2.56)	(1 : 3.05)
Age at onset (years)[a]	35.0±13.9	34.4±18.0	30.2±11.7	30.2±17.8	NA
Age at blood sampling (years)[a]	44.9±12.4	40.6±16.2	37.4±10.7	39.3±15.2	43.5±12.6
Disease duration at blood sampling (months)[a]	109.2±97.4	75.1±70.5	82.9±100.5	101.3±98.5	NA
EDSS scores at blood sampling[a]	3.76±2.50	5.13±1.87 *	3.02±2.08	4.62±1.58	NA

EDSS: Kurtzke's Expanded Disability Status Scale; NA: not applicable.
[a] mean±S.D., * $p<0.05$.

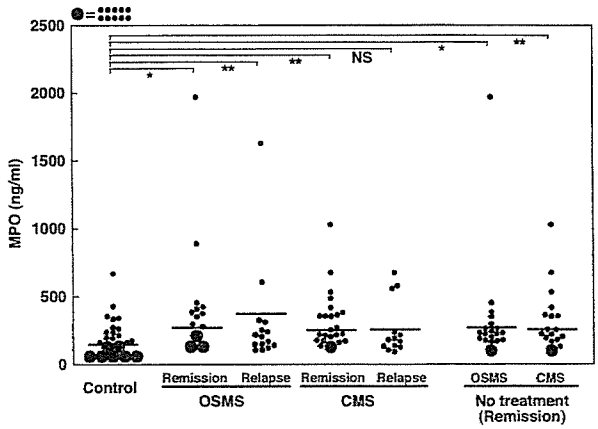

Fig. 1. Serum MPO levels in MS patients and healthy controls. MPO levels are significantly higher in OSMS patients at relapse and remission, and in CMS patients at remission compared to the controls. Moreover, even when patients undergoing low-dose corticosteroid treatment are excluded, MPO levels are still higher in OSMS and CMS patients at remission than healthy controls. Bars indicate the mean in each group. OSMS=opticospinal MS, CMS=conventional MS, NS=not significant, $*p<0.01$, $**p<0.05$.

2.4. Statistical analysis

Statistical analyses of age at onset, age at blood sampling, disease duration, EDSS score and MPO were initially performed using BMDP statistical software (BMDP Corp, CA), which includes the Kruskal–Wallis H test. If statistical significance was found, a multiple comparisons test was used to determine the statistical differences between each subgroup. Logistic regression analysis was performed to assess the association of OSMS or CMS with the MPO high (higher than the mean±2 S.D. of healthy controls) group, controlling for the following variables compared to those with the MPO normal (less than or equal to the mean±2 S.D. of healthy controls) group: gender, age at examination, age at onset, disease duration, EDSS, use of low-dose corticosteroids and presence of longitudinally extensive spinal cord lesion. In all assays, $p<0.05$ was considered statistically significant.

3. Results

3.1. MPO levels in MS patients and healthy controls

Serum MPO levels were significantly different among the five groups examined, i.e., OSMS patients at relapse, OSMS patients at remission, CMS patients at relapse, CMS patients at remission, and controls by the Kruskal–Wallis H test ($p<0.001$). According to Dunn's multiple comparison test, serum MPO was significantly higher in OSMS patients at relapse (314.7±385.0 ng/ml, $p<0.05$) and remission (269.9±311.7 ng/ml, $p<0.01$) than in healthy controls (143.8±89.7 ng/ml) (Fig. 1). CMS patients, on the other hand, had significantly higher MPO values at remission (259.4±201.9 ng/ml, $p<0.01$), but at relapse the increase was not statistically significant due to the small sample size (259.8±202.2 ng/ml, $p>0.05$). Moreover, even when patients undergoing low-dose corticosteroid treatment were excluded, MPO levels were greater in OSMS (263.7±346.4 ng/ml, $p<0.01$) and CMS patients at remission (254.6±216.4 ng/ml, $p<0.05$) compared to healthy controls.

3.2. Relationship between MPO levels and clinical and MRI parameters in MS

The MS patients were divided into MPO high (higher than the mean±2 S.D. of healthy controls) and MPO normal (less than or equal to the mean±2 S.D. of healthy controls)

Table 2
Results of logistic regression analysis to predict high versus normal MPO in (a) OSMS and (b) CMS patients at remission

(a) OSMS patients

Variables	High MPO (n=8)	Normal MPO (n=32)	Odds ratio	95% CI	p value
Gender, % female	87.5	87.5	1.66	0.08–34.74	0.7441
Mean age (S.D.) at onset, years	32.8 (12.9)	35.6 (14.3)	0.85	0.64–1.12	0.2536
Mean age (S.D.) at blood sampling, years	45.5 (13.4)	44.8 (12.3)	1.15	0.88–1.52	0.3045
Mean duration (S.D.) of disease, years	11.7 (8.7)	8.5 (8.0)	0.82	0.60–1.12	0.2188
Mean EDSS score (S.D.)	5.75 (2.30)	3.27 (2.32)	1.77	1.08–2.91	0.0245
Low-dose corticosteroids, %	50.0	25.0	2.94	0.28–31.40	0.3726
LESCL, %	62.5	56.7	0.59	0.07–5.34	0.6378

(b) CMS patients

Variables	High MPO (n=9)	Normal MPO (n=23)	Odds ratio	95% CI	p value
Gender, % female	73.9	66.7	0.92	0.08–11.31	0.9477
Mean age (S.D.) at onset, years	37.1 (6.7)	27.5 (12.3)	1.74	0.53–5.72	0.3596
Mean age (S.D.) at blood sampling, years	42.8 (5.3)	35.3 (11.6)	0.61	0.19–2.01	0.4178
Mean duration (S.D.) of disease, years	5.8 (6.7)	7.4 (9.0)	1.60	0.53–4.87	0.4060
Mean EDSS score (S.D.)	3.50 (2.38)	2.83 (1.98)	1.01	0.58–1.75	0.9856
Low-dose corticosteroids, %	22.2	17.4	1.75	0.09–34.50	0.7140
LESCL, %	12.5	40.0	0.34	0.02–6.66	0.4768

EDSS: Kurtzke's Expanded Disability Status Scale; LESCL: longitudinally extensive spinal cord lesions; CI: confidence interval.

groups, and the clinical parameters were compared between the two by logistic regression analysis. In OSMS, among the variables, only EDSS was associated with high MPO (OR=1.77 [95% CI=1.08 to 2.91], p=0.0245) (Table 2). On the other hand, no variables were associated with high MPO in CMS. Although LESCL were found in 54.5% of OSMS patients and 37.1% of CMS patients (p=0.12), the frequency of LESCL was not associated with MPO level in either of the MS subgroups by logistic regression analysis.

4. Discussion

In the present study, we observed a significant increase in serum MPO in both OSMS and CMS patients. Moreover, in OSMS patients, a high MPO level showed a significant association with greater EDSS scores by logistic regression analysis. All these findings strongly suggest that MPO levels are related to severe tissue destruction in MS patients, especially those with OSMS.

MPO is produced and secreted by granulocytes and macrophages/monocytes; therefore, serum MPO levels are thought to reflect the activities of these myeloid cells. Thus, the results of our study indicate hyperactivity of these cells in MS. However, these findings are not in accord with those of Ramsaransing et al. (2003), who directly measured the MPO activity of isolated peripheral blood leukocytes and found low leukocyte MPO activity in Caucasian patients with benign and progressive MS. There are several possibilities that could account for this discrepancy, i.e., distinct ethnicity (Caucasians vs. Asians) and subtype (classical type MS vs. OSMS), the different components measured (leukocytes vs. serum) and the distinct methods applied (bioactivity measure vs. enzyme immunoassay). Especially, high MPO was preferentially found in OSMS patients with high EDSS scores, which are rare in Caucasians and were probably not included in Ramsaransing's series; thus, the distinct subtypes used in the two studies may in part explain the apparent discrepancy between our results and those of Ramsaransing et al.

The genetic association between MPO polymorphisms and MS is also controversial. Chataway et al. (1999) suggested a possible linkage between the MPO gene and MS susceptibility, while Nagra et al. (1997) described that the high producer genotype of the MPO gene promoter region is significantly associated with early onset MS in females. Recently, Zakrzewska-Pniewska et al. (2004) also described that the high producer genotype is associated with higher EDSS scores and secondary progression. Contrarily, Kantarci et al. (2000) and Nelissen et al. (2000) both failed to find an association between the MPO gene and MS susceptibility. Our study in Japanese supports the notion that MPO contributes to tissue damage and severe residual disability in MS; therefore, further genetic study of MPO gene polymorphisms seems warranted in Asians.

We previously demonstrated that MPO-positive granulocytes are heavily infiltrated in destructive spinal cord lesions in OSMS (Ishizu et al., 2005). Granulocytes have also been shown to be associated with severe tissue damage in autoimmune diseases such as rheumatoid arthritis (Kotake et al., 1999; Ziolkowska et al., 2000; Miossec, 2003). Moreover, we recently reported that the IL-17/IL-8 axis is activated in OSMS patients, and that IL-8, which is known to be chemotactic for granulocytes and to increase myeloperoxidase and elastase activity (Laan et al., 1999; Hoshino et al., 2000; Linden and Adachi, 2002; Miyamoto et al., 2003; Witowski et al., 2004), was positively correlated with EDSS score (Ishizu et al., 2005). This finding is in good agreement with the present observations that MPO levels were positively correlated with EDSS scores in OSMS patients. All these findings suggest the possibility that activation of granulocytes is one of the key factors determining the degree of tissue destruction and thus disability in OSMS patients, although the disease is triggered by autoreactive Th_1 cells. On the other hand, serum MPO levels were significantly higher in CMS patients at remission than in healthy controls, while EDSS was not associated with high MPO. We previously reported that infiltration of neutrophils was not evident in the spinal cord lesions of CMS (Ishizu et al., 2005). Thus, although activation of neutrophils is increased peripherally in CMS patients, it may not contribute to intrathecal lesion formation in CMS. Further studies on effector cells are required to clarify the distinct roles of MPO and neutrophils in each subtype.

MPO is also positive in macrophage/microglial cells infiltrated and activated in MS plaques (Nagra et al., 1997). In OSMS patients, there is especially heavy infiltration of macrophages into lesions (Ishizu et al., 2005) and both macrophage migration inhibitory factor (Niino et al., 2000) and macrophage inflammatory protein-1β (Ishizu et al., 2005) are higher in the CSF of OSMS compared with CMS patients. Thus, macrophages might be an alternative source of increased MPO in OSMS.

In summary, the present study indicated that MPO levels are related to severe tissue destruction and thus disability in MS patients, especially those with OSMS. This probably reflects high granulocyte and macrophage activity, which is consistent with previous observations suggesting increased oxidation stress in MS. Granulocyte-blocking agents or antioxidant therapy might therefore be treatments worth investigating in MS patients.

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The selective Rho-kinase inhibitor Fasudil is protective and therapeutic in experimental autoimmune encephalomyelitis

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Abstract

We studied the role of fasudil, a selective Rho-kinase inhibitor, in experimental autoimmune encephalomyelitis (EAE). Both parenteral and oral administration of fasudil prevented the development of EAE induced by proteolipid protein (PLP) p139-151 in SJL/J mice. Specific proliferation of lymphocytes to PLP was significantly reduced, together with a downregulation of interleukin (IL)-17 and a marked decrease of the IFN-γ/IL-4 ratio. Immunohistochemical examination also disclosed a marked decrease of inflammatory cell infiltration, and attenuated demyelination and acute axonal transaction. These results may provide a rationale of selective blockade of Rho-kinase by oral use of fasudil as a new therapy for multiple sclerosis.

Keywords: Fasudil; Rho-kinase; Multiple sclerosis; Experimental autoimmune encephalomyelitis; IL-17

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS), in which an autoimmune attack is supposed to be mediated by myelin antigen-specific Th1 cells. Increasing evidence suggests that statins, which downregulate cholesterol synthesis through inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, have anti-inflammatory effects and are protective in animal models of MS, experimental autoimmune encephalomyelitis (EAE) (Aktas et al., 2003; Greenwood et al., 2003; Nath et al., 2004; Stanislaus et al., 1999; Youssef et al., 2002). Moreover, a recent open trial of simvastatin for MS revealed a significant reduction in the number of new brain lesions on magnetic resonance imaging (Vollmer et al., 2004). Nearly all of statins' pleiotropic effects on immune system are reversed by L-mevalonate, indicating that the inhibition of mevalonate pathway is crucial. Although the exact mechanism of the protective effects of statins in EAE or MS is still unclear, it is in part attributable to the prevention of isoprenylation of Rho GTPase, which occurs downstream of mevalonate pathway and is required for the membrane translocation and activation of Rho GTPase proteins (Neuhaus et al., 2004). Thus, statins may inhibit cellular functions of various cell types, including immunocytes, by inducing accumulation of the inactive form of Rho in the cytosol and thereby inhibiting downstream Rho-kinase/ROK/ROCK.

It has been reported that daily injections of protein prenyltransferase inhibitors, which prevent functional Rho GTPase, before disease onset attenuated the severity of EAE but failed to treat the disease when injections started after the onset of symptoms (Walters et al., 2002). Recently, flavonoids have also been shown to be protective in EAE through downregulating Rho GTPase activity (Hendriks et al., 2004). It is therefore

Abbreviations: APP, amyloid precursor protein; ERM, ezrin/radixin/moesin; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; MBP, myelin basic protein; NF, neurofilament; PLP, proteolipid protein.

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Table 1
Fasudil ameliorates the development of acute EAE

Treatment	n	EAE incidence (%)	Mean maximum clinical score	Mean day of onset
Intraperitoneal administration				
Vehicle	9	100.0	2.33	13.3
Fasudil	7	42.9[a]	1.00	12.7
Oral administration				
Vehicle	8	100.0	2.88	12.9
Fasudil	11	54.5[a]	1.64	12.7

[a] $p<0.05$, significantly different from vehicle-treated control group.

critical to know whether direct inhibition of Rho-kinase may have a protective effect on EAE.

On the other hand, since cholesterol is essential for myelination (Saher et al., 2005) and neurite growth and maintenance (Schulz et al., 2004), cholesterol-lowering effects of statins may be unfavorable for MS. We therefore studied the effects of the specific inhibitor of Rho-kinase fasudil, which has been safely used for vasospasm following subarachnoid hemorrhage since 1995 in Japan (Tachibana et al., 1999), in EAE. In this report, we demonstrate that fasudil acts both in a preventive and therapeutic fashion in EAE through a down-regulation of IL-17 producing T cells and Th1 cells.

2. Materials and methods

2.1. Animals

Female SJL/J mice, 6–7 weeks old, were purchased from Charles River Japan Inc. All animal protocols were approved by the Committee on Ethics in Animal Experiments of the Kyushu University and were performed according to the Guidelines for Animal Experiments of the Kyushu University and of the Japanese Government.

2.2. Antigen and antibodies

The PLP peptide p139-151 (HSLGKWLGHPDKF) was synthesized using a peptide synthesis system (Applied Biosystems), based on the 9-fluorenylmethyloxycarboneyl (Fmoc) strategy and purified by C18 reverse-phase HPLC. The purity of the peptide was >95% as determined by HPLC analysis (Minohara et al., 2001). The following primary antibodies were used for immunohistochemistry and Western blot analysis: anti-ERM antibody, anti-Phospho-ERM antibody (Cell Signaling Technology), anti-MBP antibody (Acris Antibodies, Germany), anti-mouse CD45 antibody (BD Biosciences), anti-NF 200 kD antibody and anti-APP antibody (Chemicon).

2.3. Induction and clinical evaluation of EAE in SJL/J mice

EAE was induced in SJL/J mice by immunization with 200 μg PLP p139-151 emulsified in an equal volume of complete Freund's adjuvant containing 4 mg/ml of heat-killed mycobacterium tuberculosis H37Ra (Difco). The PLP emulsion (0.1 ml) was injected subcutaneously in both sides of the rear flank. 200 ng of pertussis toxin (Sigma–Aldrich) was given intraperitoneally at the time of immunization and 48 h later. Every day, mice were weighed and examined for clinical signs of EAE and scored as follows: 0, normal; 1, limp tail; 2, impaired rightening reflex; 3, partial hind limb paresis; 4, total hind limb paralysis; 5, moribund or death.

2.4. Fasudil treatment

Fasudil (Asahi Chemical Industries, Tokyo, Japan) was administered orally or intraperitoneally. For oral treatment, fasudil was then dissolved in the drinking water to reach a

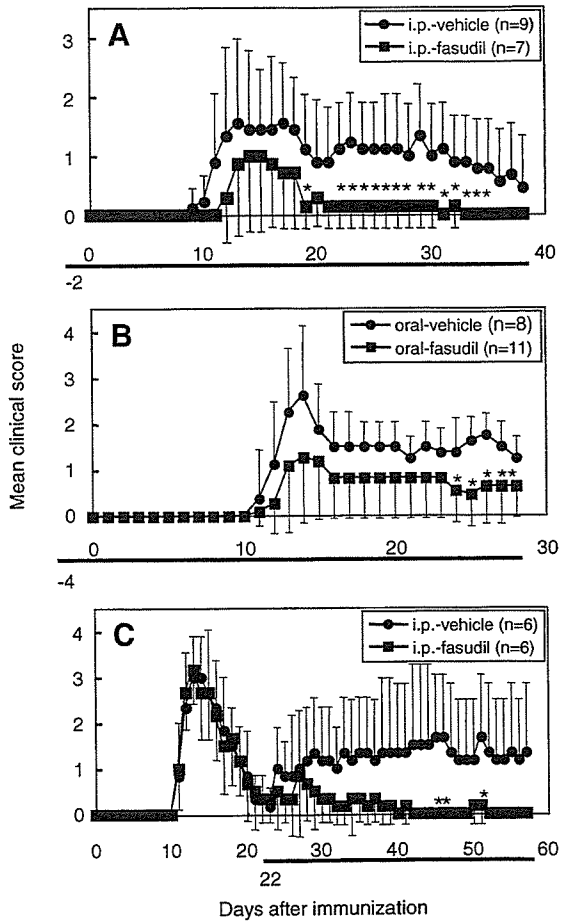

Fig. 1. Fasudil treatment inhibits acute and relapsing EAE. (A) Mice sensitized for EAE were injected intraperitoneally with fasudil (50 mg/kg/day) from day-2. EAE incidence and severity were significantly reduced in fasudil-treated mice ($n=7$) compared with vehicle (PBS)-treated controls ($n=9$) (*, $p<0.05$). (B) Oral fasudil administration ($n=11$) from day −4 also significantly prevented occurrence of EAE and decreased its severity compared with vehicle-treated mice ($n=8$) (*, $p<0.05$). (C) When fasudil treatment started at day 22, relapses were greatly inhibited and clinical symptoms showed significant alleviation after day 45 (*, $p<0.05$) compared with vehicle-treated mice ($n=6$ in each group). Data are presented as mean± SD of clinical scores.

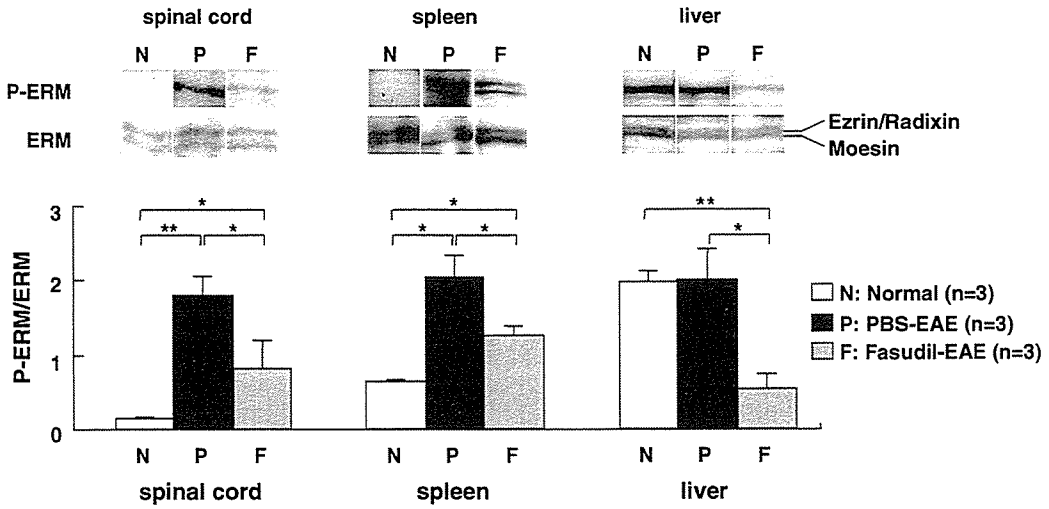

Fig. 2. Rho-kinase activity in EAE. Western blot analysis for phosphorylated ERM, a marker of Rho-kinase activity, normalized by total ERM, in spinal cord (day 14), spleen (day 10) and liver (day 10) of normal mice and of PLP-induced EAE mice. In spinal cord and spleen, ERM phosphorylation was significantly increased in PBS-treated EAE mice as compared with normal mice, which was suppressed by the fasudil treatment. (*, $p<0.05$; **, $p<0.01$). In liver, ERM phosphorylation was also suppressed by fasudil treatment as compared with PBS-treated EAE mice and normal mice. Results are expressed as mean±SEM.

final dosage of 100 mg/kg/day (Higashi et al., 2003) and the treatment was started 4 days before antigen immunization. To adjust the daily intake of fasudil, we measured the water intake and body weight on a daily basis. The fasudil-treated mice were able to freely access the water in which fasudil was dissolved. Mice with normal drinking water served as control. For intraperitoneal treatment, fasudil suspended in phosphate buffered saline (PBS) (50 mg/kg) was administered daily from 2 days before antigen immunization while PBS alone was administered as a control.

2.5. Antigen-specific T cell proliferation assays

Splenocytes or LN cells were harvested and processed into single cell suspensions. Cells (2×10^5 cells/well) were distributed into 96-well round-bottom plates (Falcon) and cultured with PLP p139-151 (0.1, 0.5, 1, 5, 10 μM), PHA (10 μg/ml), or medium alone. After 48 h of culture, 1 μCi of [^{3}H] thymidine was added per well and cultures were harvested 18 h later and assessed for incorporation of [^{3}H] thymidine. All assays were performed in triplicate.

2.6. Cytokine analysis

The supernatant from cultures of splenocytes and LN cells was harvested at 72 and 120 h: 72 h for IL-1β, IL-2, GM-CSF, IFN-γ, TNF-α and IL-17, and 120 h for IL-4, IL-5 and IL-10. Levels of IL-1β, IL-2, GM-CSF, IFN-γ, TNF-α, IL-4, IL-5 and IL-10, IL-17 were simultaneously analyzed using the Bio-Plex Cytokine Assay System (Bio-Rad Laboratories) according to the manufacturer's instructions as described previously (Ishizu et al., 2005). All assays were performed in triplicate.

2.7. Western blot analysis for ERM phosphorylation

To quantify Rho-kinase activity in the spinal cord (day 14 after immunization), liver (day 10) and spleen (day 10), Western blot analysis for phosphorylated ERM was performed by using antibodies that specifically recognize phosphorylated ERM (ezrin T567, radixin T564 and moesin T558) and total ERM as described previously (Higashi et al., 2003). ERM is phosphorylated by Rho-kinase at T567 (ezrin), T564 (radixin) and T558 (moesin). Equal amounts of extracted protein was loaded for SDS-PAGE/immunoblot analysis. The regions containing ERM family proteins were visualized by electrochemiluminescence. Band intensities from Western blots were quantified densitometrically by ImageJ 1.34s downloaded from http://rsb.info.nih.gov/ij. The extent of ERM phosphorylation was normalized by that of total ERM.

2.8. Histopathology and immunohistochemistry

Mice were anesthetized and perfused with PBS and 4% buffered paraformaldehyde. Spinal cords were collected on day 14 and 42 after antigen immunization in the preventive study, and on day 67 in the therapeutic study. The tissue was dissected and post-fixed in 4% buffered paraformaldehyde solution and embedded in paraffin. After embedding, 6-μm-thick section were prepared. For routine neuropathological evaluation, sections were stained with the hematoxylin-eosin (H–E) stain. For immunohistochemical analysis, sections were deparaffinized in xylene, hydrated in ethanol, and sections were incubated in 0.3% hydrogen peroxide in absolute methanol for 30 min at room temperature to inhibit endogenous peroxidase. After rinsing in tap water, the sections were completely immersed in distilled water and autoclaved for

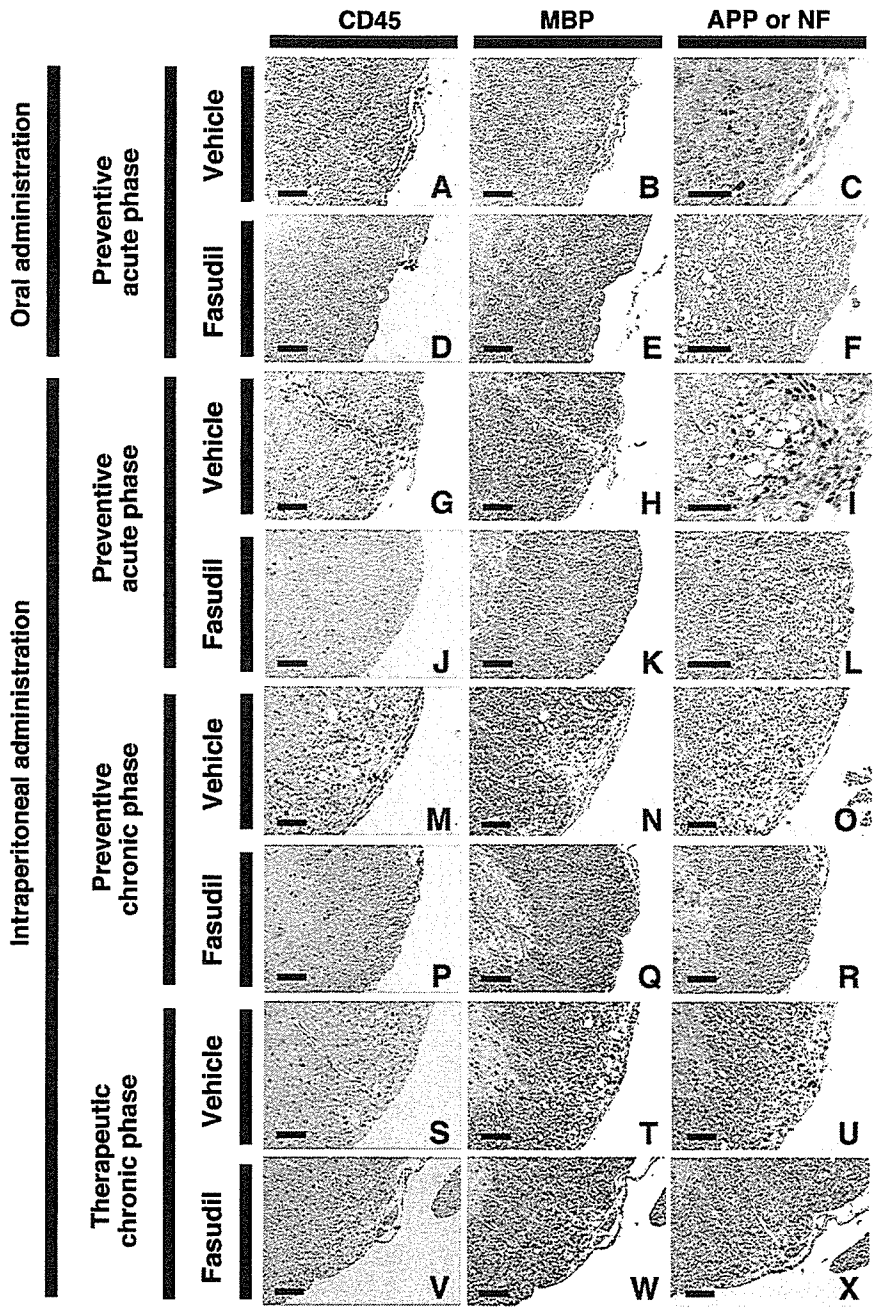

Fig. 3. Decreased inflammation, demyelination and axonal loss in spinal cord of fasudil-treated mice with EAE. Either vehicle or fasudil was given daily until the animals were sacrificed. Spinal cords lesions were assessed by immunohistochemical staining of CD45 (left), MBP (middle), APP (right, C, F, I, L) and NF (right, O, R, U, X). When fasudil administration started before immunization, in the acute phase (spinal cords collected on day 14, A to L), vehicle-treated mice showed leukocyte infiltrations at perivascular areas, parenchyma and meninges (A, G), and demyelination (B, H) and axon transection (C and I) were also evident. Meanwhile, fasudil-treated mice, both with oral (D to F) and intraperitoneal (J to L) administrations, showed a significant reduction of leukocytes infiltration (D and J), demyelination (E and K) and axon transaction (F and L). In the chronic phase (spinal cords collected on day 42, M to R), leukocyte infiltration, demyelination and axonal damage were more distinct in PBS-treated mice (M to O), but these findings were dramatically reduced in fasudil-treated ones (P to R). When fasudil administration started from day 22 after immunization, fasudil-treated mice demonstrated a significant reduction of leukocyte infiltration (V), demyelination (W) and axonal destruction (X) at day 67 compared with those of PBS-treated mice (S to U). The scale bar in A, B, D, E, G, H, J, K, M–X=50 μm, C, F, I, L=25 μm.

15 min to enhance the immunoreactivity of CD45, MBP, NF and APP. Subsequently, sections were incubated with primary antibody diluted in 5% non-fat milk in TBST (25 mM Tris–HCl pH 7.6 containing 0.5 M NaCl, 0.05%, NaN_3 and 0.05% Tween 20) for 1 h at room temperature (Kikuchi et al., 1999). As secondary antibody, biotinylated goat anti-rabbit IgG and streptavidin peroxidase or Simple Stain mouse MAX-PO (Rat) (Nichirei, Japan) were used. When mouse primary antibody

Table 2
Fasudil suppresses histological EAE[a]

Treatment	Inflammatory infiltrates[b]	Demyelination (%)[c]	Axonal loss (%)[c]	APP positivity[d]
Intraperiponeal administration before immunization				
Acute phase (day 14)				
Vehicle	1.94±0.16	1.28±0.18	0.66±0.12	16.41±3.01
Fasudil	0.82±0.15[e]	0.38±0.10[e]	0.15±0.08[e]	1.16±0.51[e]
Chronic phase (day 42)				
Vehicle	1.96±0.24	7.96±2.42	13.18±3.83	NA
Fasudil	0.20±0.09[e]	0.45±0.19[e]	0.31±0.14[e]	NA
Oral administration before immunization				
Acute phase (day 14)				
Vehicle	2.37±0.07	2.69±0.30	2.64±0.31	19.76±3.06
Fasudil	1.28±0.11[e]	0.91±0.17[e]	0.71±0.15[e]	5.83±1.81[e]
Intraperiponeal administration from day 22				
Chronic phase (day 67)				
Vehicle	2.63±0.11	8.01±0.91	10.63±1.40	NA
Fasudil	1.24±0.14[e]	2.77±0.43[e]	3.30±1.16[e]	NA

NA: not applicable.
[a] A total of 3–4 mice per group, and 12 spinal cord sections per mouse which covered the whole length of the spinal cord were examined and quantified.
[b] Inflammatory infiltrates: 0, no positive cells visible; 1, a few inflammatory cells; 2, organization of perivascular infiltrates; 3, increasing severity of perivascular cuffing with extension into the adjacent tissue.
[c] Demyelination and axonal loss were quantified as percentages of areas of demyelination and axonal loss against total white matter area examined by MBP and NF immunostaining, respectively.
[d] APP positivity was quantified by counting APP positive axons in a defined quarter of each section and calculated as positive axons per mm^2.
[e] $p<0.01$, significantly different from vehicle-treated control group.

was used, the Histofine mouse staining kit (Nichirei, Japan) was used to block endogenous mouse immunoglobulins in the tissue. The colored reaction product was developed using Simple Stain DAB solution (Nichirei, Japan). The sections were counterstained lightly with hematoxylin. In each group, 12 sections per mouse which covered the whole length of spinal cord were histologically examined and quantified. Inflammatory cell infiltrates were graded by CD45 immunostaining as: 0: no positive cells visible, 1: a few inflammatory cells, 2: organization of perivascular infiltrates, 3: increasing severity of perivascular cuffing with extension into the adjacent tissue. ImageJ 1.34s was used to calculate percentages of demyelinated areas per total white matter area examined by MBP immunostaining, and areas of axonal loss per total white matter area examined by NF immunostaining (Pluchino et al., 2003). Axonal transection was evaluated by APP immunoreactivity and quantified by counting APP positive axons in a defined quarter of each section and calculated as positive axons per mm^2.

2.9. Statistical analysis

Disease frequency was compared by Fisher's exact probability test. Ratios of phosphorylated ERM against total ERM, proliferation of T cell and cytokine production were compared by Student's *t* test. All other statistics were analysed with the Mann–Whitney *U* test. A value of $p<0.05$ was considered significant.

3. Results

3.1. Fasudil suppresses PLP-induced EAE

Intraperitoneal injection of fasudil significantly reduced incidence of EAE in SJL/J mice immunized with PLP p139-151 ($p<0.05$) (Table 1). All PBS-treated mice developed neurological symptoms with the average onset at day 13.3 and the peak at day 14 while in fasudil-treated mice, only 42.9% of mice developed neurological symptoms with the average onset at day 12.7 and the peak at day 16. The severity of the disease in fasudil-treated mice was significantly reduced at day 19 and day 22–35, as compared with PBS-treated mice ($p<0.05$) (Fig.1A). The oral administration of fasudil was examined next. According to the results of preliminary experiments on the volume of daily water intake of mice, the concentration of fasudil was determined to reach a dosage of 100 mg/kg/day. All control mice developed neurological impairment while only 54.5% of mice orally treated with fasudil developed a comparatively mild EAE (Table 1). The incidence and severity of EAE after day 24 were significantly reduced in the orally fasudil-administered group compared to vehicle controls ($p<0.05$) (Fig. 1B).

3.2. Rho-kinase activity in mice on EAE with or without fasudil

Ezrin/radixin/moesin (ERM) is one of the major substrates of Rho-kinase. To confirm that fasudil inhibited the Rho-kinase pathway *in vivo*, we measured the extent of ERM phosphorylation by Western blot analysis as a marker of the Rho-kinase activity *in vivo* in the spinal cord, liver and spleen. In the spinal cord (day 14) and spleen (day 10), the ratio of phosphorylated ERM against total ERM in PBS-treated animals showed a major increase compared with normal mice ($p<0.01$), and was inhibited significantly in fasudil-treated animals ($p<0.05$) (Fig. 2). In the liver, 10 days after antigen immunization the ratio of phosphorylated to total ERM in fasudil-treated animals also decreased significantly compared with PBS-treated animals and normal animals ($p<0.05$). These data indicate that Rho-kinase activity is up-regulated in the spinal cord and spleen upon EAE and fasudil markedly suppresses its activity *in vivo*.

3.3. Fasudil decreases infiltration of inflammatory cells into the CNS

Histopathological examination of the preventive study (administration before immunization) revealed moderately damaged lesions in the white matter of the spinal cord in the acute phase of the vehicle group at the peak of disease progression (day 14) (Fig. 3A to C, G to I and Table 2). In this group, CD 45 positive inflammatory cells infiltrated the

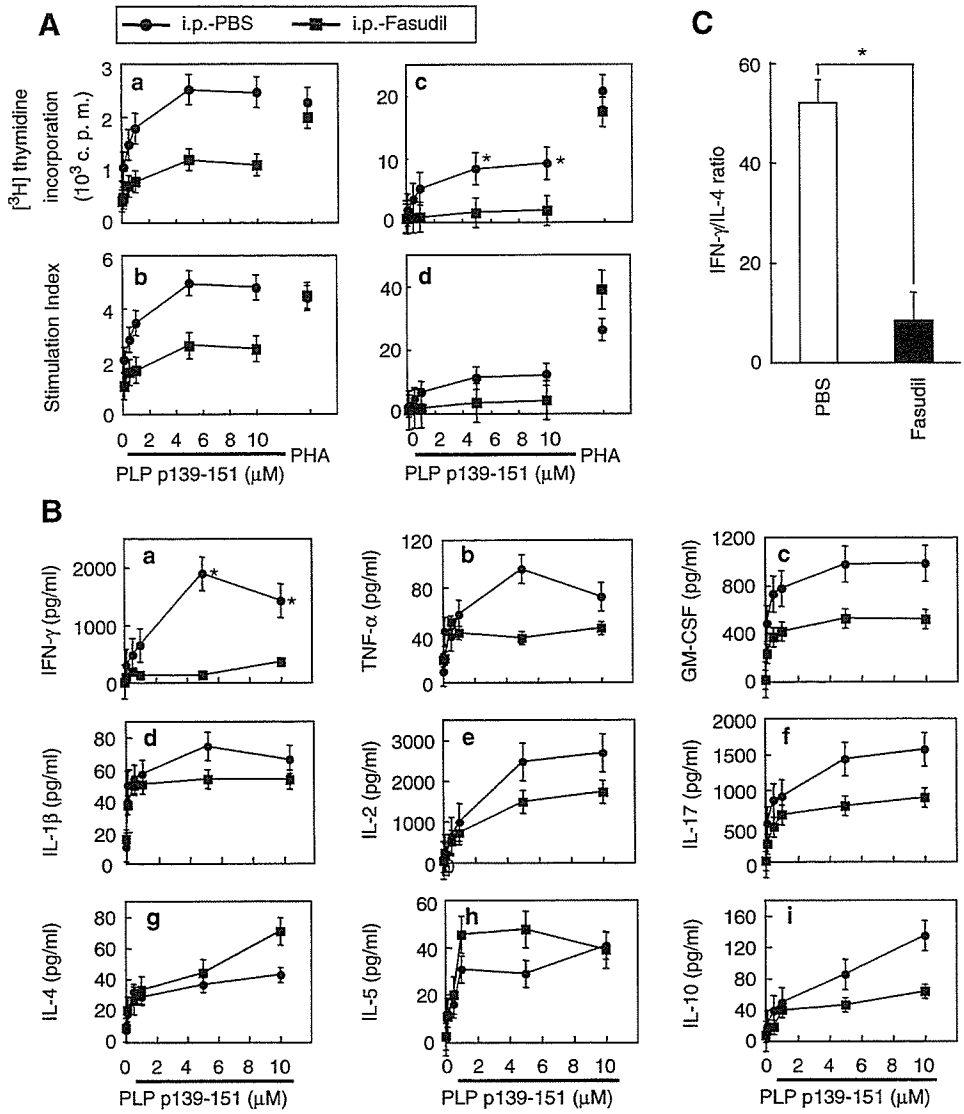

Fig. 4. Fasudil suppressed antigen-dependent proliferation of lymphocytes and induced a Th2 cytokine bias in PLP-induced EAE mice. Splenocytes and LN cells were harvested from fasudil-treated (n=3) or PBS-treated (n=3) mice at day 10 after immunization. The cells were cultured with PLP p139-151, PHA or medium alone. T cell proliferation was determined by [^{3}H] thymidine incorporation. Cytokines were measured by Bio-Plex Cytokine Assay System and ELISA. (A) PLP p139-151-specific proliferation of splenocytes (a, b) and LN cells (c, d) were markedly reduced in fasudil-treated mice (closed square) compared with PBS-treated mice (closed circle). (B) Cytokine assay of supernatants from splenocyte culture with PLP p139-151. Fasudil treatment was associated with a marked reduction in secretion of IFN-γ (a) ($p<0.05$) and a moderately reduction of IL-17 (f), TNF-α (b), GM-CSF (c), IL-1β (d), IL-10 (i), IL-2 (e) secretion. In contrast, secretion of IL-4 (g) and IL-5 (h) was slightly increased. (C) The IFN-γ/IL-4 ratio in the culture supernatant of splenocytes from PLP-sensitized mice stimulated with PLP p139-151 greatly decreased in fasudil-treated mice compared with PBS-treated mice ($p<0.05$). Data shown are mean±SE of a group (n=3).

demyelinated areas (Fig. 3A, G) and numerous amyloid precursor protein (APP)-positive transected axons were visible (Fig. 3C, I) in the lesions. Neurofilament (NF) immunostaining was relatively preserved in this phase (Table 2). In contrast, inflammatory infiltrated cells, demyelinated areas and axonal transactions were dramatically recovered in the fasudil treated groups with both oral (Fig. 3D to F) and intraperitoneal administration (Fig. 3J to L) as compared with the vehicle group (Table 2). In the chronic phase, inflammatory infiltrates (Fig. 3M, P) were significantly reduced in fasudil-treated mice as compared with PBS-treated mice, both myelin basic protein (MBP) (Fig. 3N, Q) and NF (Fig. 3O, R) immunostaining severely decreased in PBS-treated mice but not in fasudil-treated mice (Table 2). These findings indicated that fasudil suppresses inflammatory cell infiltration, demyelination and acute axonal transection in the CNS.

3.4. Fasudil treatment induces a reduction of PLP-specific T cell proliferation and a Th2 cytokine bias

To gain insights into the mechanisms of the inhibitory effects of fasudil, splenocytes and lymph node (LN) cells

were removed from fasudil-treated and PBS-treated mice on day 10, and the cells were restimulated with PLP p139-151 at different concentrations and their cytokine production and antigen-specific proliferation were measured. Fig. 4A shows the results of the proliferation of splenocytes and LN cells, which indicated that treatment of mice with fasudil suppressed the proliferative response to the antigen. Inhibition of proliferation appeared to be dose-related (LN cells: $p<0.05$). Culture supernatants were then examined for cytokines. As shown in Fig. 4B, fasudil treatment was associated with a marked reduction in secretion of IFN-γ ($p<0.05$) and a moderately reduction of IL-17, TNF-α, GM-CSF, IL-1β, IL-10, IL-2 secretions. In contrast, secretion of IL-4 and IL-5 was slightly increased. As a result, the IFN-γ/IL-4 ratio in the supernatant was strongly suppressed in fasudil-treated mice as compared with PBS-treated ones ($p<0.05$) (Fig. 4C).

3.5. Fasudil treats chronic EAE when administered after disease onset

The therapeutic potential of fasudil was assessed by intraperitoneal treatment of PLP-induced EAE mice daily with 50 mg/kg fasudil from day 22 after immunization when all mice were in their first recovery phase. As shown in Fig. 1C, PBS-treated mice developed a more severe relapse than fasudil-treated mice. In fasudil-treated mice, clinical symptoms showed a significant reduction at day 45, 46, and 51 ($p<0.05$). Moreover, in this therapeutic study, histological examination (Fig.3S to X and Table 2) disclosed that demyelination was significantly reduced in the fasudil-treated mice (Fig. 3T, W and Table 2) together with decreased inflammatory cell infiltration (Fig. 3S, V and Table 2) while NF staining was significantly preserved (Fig. 3U, X and Table 2). These data demonstrate that fasudil administrated after the onset of EAE significantly reduces the development and severity of EAE through prevention of leukocyte infiltration into the CNS and rescuing myelin and axons from damage.

4. Discussion

As shown in the present study, the specific Rho-kinase inhibitor fasudil, is protective in acute and chronic EAE induced by PLP p139-151 in mice. The drug was preventive when administered before immunization and therapeutic when administered after the onset of the disease. According to previous studies, the beneficial effects of statins on EAE and MS can partly be explained by inhibition of the isoprenylation of Rho GTPase (Greenwood et al., 2003; Neuhaus et al., 2004), which results in a Th2 shift acting on both T cells and antigen-presenting cells (APC) (Aktas et al., 2003; Nath et al., 2004; Youssef et al., 2002) and an inhibition of T cell migration to the CNS acting on both T cells (Weitz-Schmidt et al., 2001) and brain endothelial cells(Greenwood et al., 2003). In accordance with this, Walters et al. (2002) demonstrated that protein prenyltransferase inhibitors partially suppressed EAE when administered before the onset of disease, whereas no therapeutic effect was found when the administration started after the onset. Flavonoids have also been shown to be protective in EAE by modulating the activity of Rho GTPase (Hendriks et al., 2004). In addition, 17β-estradiol, which is also protective in EAE through inhibition of the production and migration of encephalitogenic T cells and is neuroprotective when initiated before immunization (Offner, 2004), downregulates expression and function of Rho-kinase *in vivo* (Chrissobolis et al., 2004; Hiroki et al., 2005). Collectively, inhibition of the Rho/Rho-kinase system is considered to be protective against EAE. Based on our findings that fasudil markedly reduced relapse and protected mice from development of progressive disability even when initiated after the first episode of EAE, the direct inhibition of Rho-kinase itself appears to be more beneficial in the progression of inflammatory demyelination of the CNS. The effects of Rho-kinase inhibitors have been shown to be more evident in males than in females (Chrissobolis et al., 2004), partly reflecting the suppressive effects of estrogen on Rho-kinase (Hiroki et al., 2005); however, in our study, female SJL/J mice reacted well to fasudil in EAE.

The Rho family proteins of small GTPases (Rho, Rac and Cdc42) act as key regulators of the actin cytoskeleton. Rho-kinase is the major effector molecule for a variety of functions of Rho GTPase (Amano et al., 2000), although several other proteins have also been identified as effectors of Rho, including protein kinase N, rhophilin, rhotekin, citron, p140mDia and citron kinase (Hall, 1998; Kaibuchi et al., 1999). Activation of Rho-kinase by GTP-bound Rho (the activated form) leads to phosphorylation of ERM, myosin light chain, collapsin response mediator protein-2 (CRMP-2), LIM kinases 1 and 2, adducin and intermediate filament (Fukata et al., 2001; Shimokawa and Takeshita, 2005). Direct inhibition of Rho-kinase activity induced suppression of cell proliferation and motility. For immune cells, Rho-kinase inhibitor has been shown to suppress T cell proliferation (Tharaux et al., 2003) and traffic (Bardi et al., 2003) and down-modulate both Th1 and Th2 cytokine production (Aihara et al., 2003). Furthermore, Rho-kinase inhibitor suppressed chemotaxis of neutrophils and eosinophils (Adachi et al., 2001; Alblas et al., 2001; Niggli, 1999). In the present study, specific proliferations to PLP p139-151 of LN cells and splenocytes were significantly suppressed in the fasudil-administered animals and the production of IL-17 and Th1 cytokines were severely inhibited while Th2 cytokines, such as IL-4 and IL-5, were slightly enhanced, resulting in a marked decrease of the IFN-γ/IL-4 ratio. These findings are consistent with the report of another Rho-kinase inhibitor, Y-27632, that strongly suppressed the production of IFN-γ but suppressed only weakly IL-4 and IL-5 in human peripheral blood T cells (Aihara et al., 2003). Therefore, in addition to a direct inhibition on leukocyte proliferation, a pronounced Th2 shift in fasudil-treated animals may well be beneficial for EAE. Yet, the production of the anti-inflammatory cytokine IL-10 was also suppressed in our study. Moreover, increasing evidence suggests that IL-17