the CSF of MS patients, but not of NMO patients, was lower than that of healthy subjects [8]. Recently, it was also reported that autoantibodies taken from North American NMO and Japanese OS-MS, NMO-IgG, stained the perivascular and pial structures of the brain tissue [9].

In the present study, we examined the TCR repertoire in PBL of Japanese patients with NMO with CDR3 spectratyping and compared the results with those from MS patients and healthy subjects. By this analysis, we determined Vβs that were frequently activated in the three groups and obtained the following intriguing findings. First, it was demonstrated that T cell immunity in NMO patients was significantly up-regulated than MS patients and healthy subjects. Second, the detailed analysis revealed that Vβ1 and Vβ13 genes were frequently used in NMO compared with MS. Third, we evaluated the longitudinal changes of the TCR repertoire and found that in NMO and relapsing-remitting MS (RRMS), the increased number of clonally expanded Vβs correlated with disability evaluated with the Kurtzke Expanded disability status scale (EDSS) [10], whereas, secondary progressive MS (SPMS) patients with longer disease durations and higher EDSS scores had a decreased number of clonally expanded Vβs than RRMS patients. These findings suggest that detailed TCR investigations will provide useful information for the recognition of the clinical and immunological status of NMO and MS and the development of effective antigen-specific and prophylactic immunotherapies.

2. Materials and methods

2.1. Patients and specimens

Eleven patients with NMO diagnosed according to the criteria [3] and 21 patients with MS who had a clinically confirmed diagnosis [11], attending the Department of Neurology, Tokyo Metropolitan Neurological Hospital, were examined. All patients with NMO were evaluated with brain magnetic resonance imaging (MRI) using a 1.5 Tesla scanner and they showed no evidence of cerebral demyelination. MS patients were classified to one of the two clinical courses of MS: RRMS or SPMS at the time of blood sampling [12]. Thirteen patients had RRMS and eight had SPMS. All of the SPMS patients had observation periods of more than eight years. All patients with NMO had relapsing-remitting course. Clinical neurological dysfunction was determined on the basis of EDSS [10]. Samples examined during relapse were taken before intravenous methylprednisolone treatment. Maintenance therapy of each patient was shown in Table 1. Eight of 11 NMO patients and 4 of 21 MS patients were taking oral prednisolone. As Interferon-β-1b and azathioprine were not used to NMO and rarely used to MS patients, the effects of immune-mediated therapies other than oral prednisolone were weak in this study. In parallel, a total of 25 healthy volunteers, 14 males and 11 females, were examined as healthy control subjects. Consent was obtained from all the subjects and the study was approved by the Institute Review Board. Fifteen milliliters of heparinized blood was drawn from the patients, and peripheral blood lymphocytes (PBL) were isolated using the density gradient method.

We also made longitudinal studies using PBL from patients with NMO (6 patients), RRMS (6 patients) and SPMS (6 patients). A total of 47 blood samples, 14 samples obtained from 6 NMO, 17 from 6 RRMS and 16 from 6 SPMS, were examined at a mean interval of 4.1±3.8 months and with a mean follow-up period of 7.6±4.7 months. Healthy subjects were also evaluated using the same method. Total of 32 blood samples from 11 healthy subjects were examined at a mean interval of 5.2±9.5 months and with a mean follow-up period of 16.2±20.8 months.

2.2. cDNA synthesis and PCR amplification

RNA was extracted from PBL using RNAzol B (Biotecx Lab, Houston, TX) or TRIzol (Invitrogen, Tokyo, Japan).

Table 1
Clinical characteristics and maintenance therapy of NMO, RRMS, SPMS patients and healthy control subjects [a]

Characteristics	NMO (n=11)	RRMS (n=13)	SPMS (n=8)	Healthy subjects (n=25)
Age (mean±S.D., years)	52.5±17.0 [b]	38.6±10.6 [b]	39.9±7.2 [b]	37.4±9.9 [b]
Male/female	2:9	4:9	5:3	14:11
Duration of disease (mean±S.D., months)	94.8±101.6	35.4±47.5 [c]	168.7±79.2 [c]	–
EDSS (mean±S.D.)	4.2±2.9 [d]	4.0±2.2 [d]	7.3±1.2 [d]	–
Maintenance therapy				
None	3	9	3	–
Oral prednisolone	8	3	1	–
Interferon-β-1b	0	0	3	–
Azathioprine	0	1	1	–

[a] Eleven NMO, 21 MS patients (13 RRMS and 8 SPMS) and 25 healthy control subjects were examined. Student's t-test or Mann–Whitney's U-test was used for the statistical analysis of the age, duration of disease and EDSS (where appropriate). The sex ratio was analyzed using the Fisher's exact probability method.

[b] Mean age of NMO patients was significantly older than that of RRMS, SPMS and healthy subjects (p=0.03, p=0.04, p=0.02, respectively).

[c] There was no significant difference in disease duration between NMO and MS. However, in the MS group, SPMS patients had significantly longer disease duration than RRMS patients (p=0.003).

[d] SPMS had significantly higher EDSS score than NMO (p=0.007) and than RRMS (p=0.0002).

cDNA was synthesized using reverse transcription with ReverTra Ace-α (Toyobo, Osaka, Japan), then amplified in a thermal cycler (Takara, Tokyo, Japan) using primer pairs for TCR. Cycling conditions for PCR were as follows: 94 °C for 1 min for denaturation and hot start, followed by 40 cycles of 98 °C for 10 s, 55 °C for 30 s, and 72 °C for 1 min for annealing and extension. TCR primers for Vβ1-24 were the same as those used in a previous study [13]. The Cβ primer was labeled with rhodamine.

2.3. CDR3 spectratyping

CDR3 spectratyping was performed as described previously [14] with a few modifications. cDNA was amplified with Vβ specific primers and rhodamine-labeled Cβ inner primers, and undiluted PCR products were added to an equal volume of formamide/dye loading buffer and heated at 95 °C for 3 min for denaturing. Six microliters of the samples were applied to a 6% acrylamide sequencing gel. Gels were run at 40 W for 2 h at 50 °C. Then, the fluorescence-labeled DNA profile on the gel was recorded directly using an FMBIO-II fluorescence image analyzer (Hitachi, Yokohama, Japan). Spectratype expansion was evaluated by inspecting visually and a density analysis of the image was performed, if necessary, using software attached to the fluorescence image analyzer and graded into two categories; the "oligoclonal" and the "monoclonal" type of spectratype expansion, as described previously [15]. The presence or absence of contamination of the reagents used in PCR was examined every 10 PCR analyses by performing PCR without the templates. When contamination was present, all reagents used and results obtained during the period were discarded.

2.4. Statistical analysis

Student's t-test or Mann–Whitney's U-test was used for the statistical analysis of the age, duration of disease, EDSS and the number of clonally expanded Vβs that each subject had (where appropriate). The sex ratio and the frequency of Vβ expansion were analyzed using Fisher's exact probability method. Correlations were assessed with the Spearman's product moment method. All statistical analyses were performed using a commercial software package (SPSS version 10.0J, SPSS Japan Inc, Tokyo, Japan).

3. Results

3.1. The representative spectratyping profiles from NMO patients and healthy subjects

The CDR3 spectratyping profile was divided into two categories, i.e. the normal pattern and the expanded pattern (Figs. 1 and 2, respectively). In the normal pattern found in a healthy control subject, each spectratype consisted of five to seven bands showing a Gaussian distribution without any spectratype expansion (Fig. 1A). The density of the bands

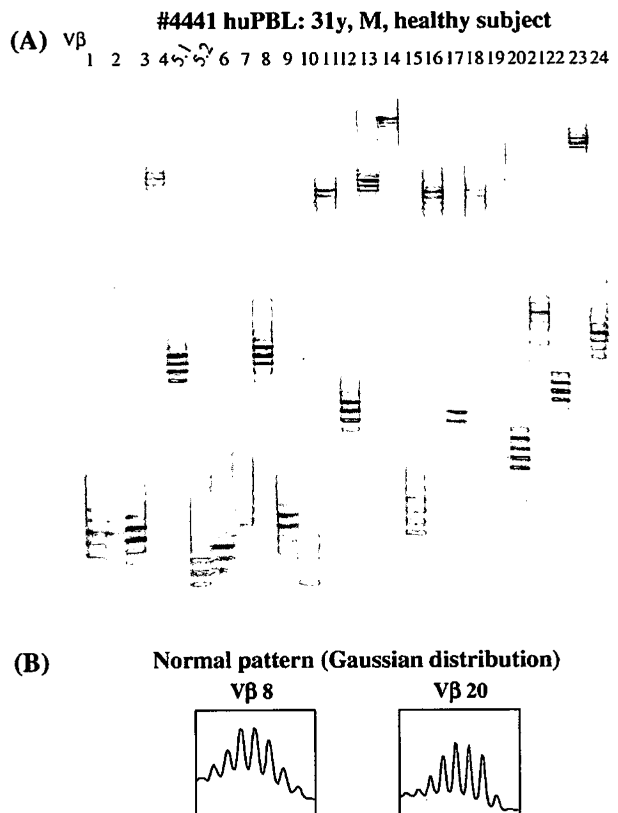

Fig. 1. (A) The normal spectratype pattern obtained from a healthy control subject. Each Vβ spectratype consists of five to seven bands and shows a Gaussian distribution in which the density of bands is generally higher in the middle part of the spectratype. (B) Density analysis of Vβ8 and Vβ20 shows the normal spectratype pattern with a typical Gaussian distribution.

was generally higher in the middle part of the bands (Fig. 1B). Fig. 2 demonstrates the expanded pattern found in an NMO patient where there were 10 expanded Vβ genes, i.e. Vβ1, Vβ2, Vβ3, Vβ4, Vβ10, Vβ13, Vβ14, Vβ16, Vβ21 and Vβ23 (arrows in Fig. 2A). For example, density analysis of Vβ10 revealed the monoclonal-type-expansion with marked increase of the density and thickness of a band with faint additional spectratypes, and Vβ16 shows the oligoclonal-type-expansion with the increase of density accompanied with the distorted Gaussian distribution (Fig. 2B). In previous studies on MS and its animal model, we determined the CDR3 sequences of TCR clones derived from oligoclonally expanded spectratypes and confirmed that expanded spectratypes represented the clonal expansion of particular TCR clones [4,14].

We defined the number of clonally expanded Vβ genes each subject had as "the number of clonally expanded Vβs". For example, "the number of clonally expanded Vβs" of the NMO patient in Fig. 2 was 10. We also defined, for each Vβ gene, the percentage of subjects who had clonal expansion of that Vβ gene in a certain group as "the frequency of Vβ expansion". For example, 5 of 11 NMO patients had the clonally expanded Vβ1 gene, so "the frequency of Vβ expansion" of Vβ1 in NMO was 45% (Fig. 4A, B).

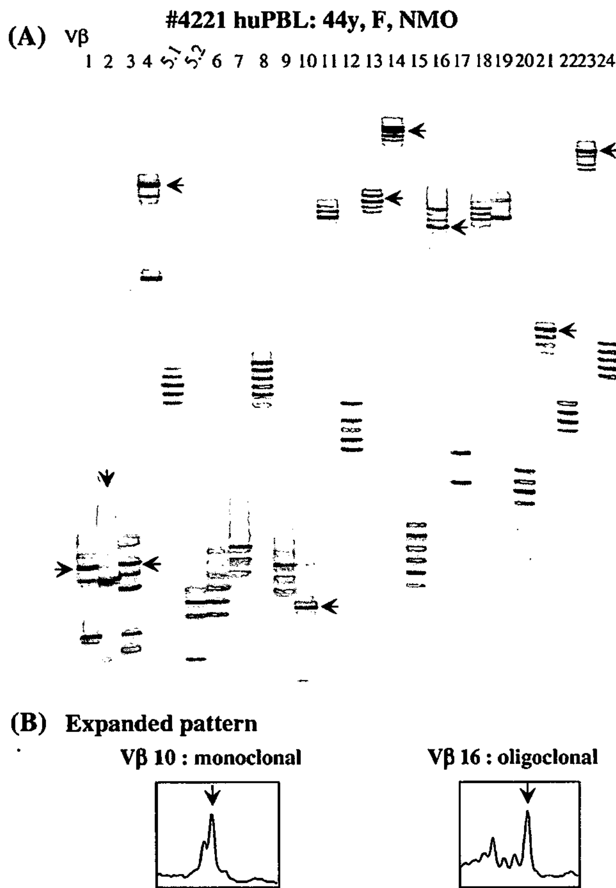

Fig. 2. (A) The spectratype pattern obtained from an NMO patient (44-year-old female). There are 10 oligoclonally expanded Vβ genes, i.e. Vβ1, Vβ2, Vβ3, Vβ4, Vβ10, Vβ13, Vβ14, Vβ16, Vβ21 and Vβ23 (arrows). (B) Density analysis of the expanded spectratype pattern. Vβ10 shows the monoclonal-type-expansion with marked increase of the density and thickness of a band with faint additional spectratype while Vβ16 exhibits the oligoclonal-type-expansion with the increase of density accompanied with the distorted Gaussian distribution.

3.2. T cell immunity was increased in NMO and decreased in SPMS

The clinical features of 11 NMO, 21 MS patients (13 RRMS and 8 SPMS) and 25 healthy individuals subjected for the cross-sectional study are summarized in Table 1. Mean age of NMO patients was 52.5±17.0 years old and they were significantly older than RRMS, SPMS and healthy subjects ($p=0.03$, $p=0.04$, $p=0.02$, respectively). Duration of the disease is not different between NMO and MS although, in the MS group, SPMS had significantly longer disease duration than RRMS ($p=0.003$). Moreover, SPMS patients had significantly higher EDSS score than NMO ($p=0.007$) and RRMS ($p=0.0002$) patients.

CDR3 spectratyping analysis was performed and the number of clonally expanded Vβs was compared among 11 NMO, 21 MS and 25 healthy subjects (Fig. 3A). Mean numbers of clonally expanded Vβs were 6.0±3.2 in NMO, 2.5±2.7 in MS and 0.9±1.1 in healthy subjects. These examinations revealed that both NMO and MS patients had a significantly larger number of clonally expanded Vβ genes than healthy subjects ($p=0.0003$ and $p=0.02$, respectively). Moreover, NMO patients had a significantly larger number of clonally expanded Vβs than MS patients ($p=0.006$).

MS shows two clinical phenotypes with RRMS and SPMS, and the mechanism of progression in SPMS is known as the neurodegenerative processes, but not an inflammatory demyelination confirmed by neuropathology and MRI studies. All the NMO patients examined in the present study showed the relapsing-remitting course. Therefore, there is the possibility that the above mentioned increased T cell immunity in NMO is attributable to the nature of the relapsing clinical course of demyelinating disease and not to

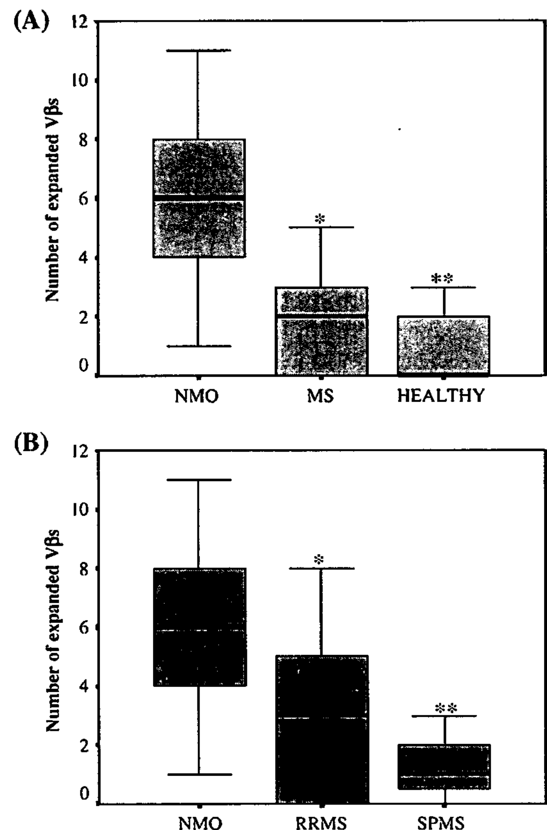

Fig. 3. The numbers of clonally expanded Vβs demonstrated by CDR3 spectratyping analysis of 11 NMO, 21 MS and 25 healthy subjects. (A) Comparison among NMO, MS and healthy subjects. Mean numbers of clonally expanded Vβs are 6.0±3.2 in NMO, 2.5±2.7 in MS and 0.9±1.1 in healthy subjects. Both NMO and MS patients has a significantly larger number of clonally expanded Vβ genes than healthy subjects ($p=0.0003$ and $p=0.02$, respectively). Moreover, NMO patients has a significantly larger number of clonally expanded Vβs than MS patients ($p=0.006$). (B) Comparison among NMO, RRMS and SPMS. Mean numbers of clonally expanded Vβs are 3.2±3.1 in RRMS and 1.3±1.0 in SPMS. Thus, NMO also has a significantly larger number of clonally expanded Vβ genes than RRMS ($p=0.04$). Furthermore, SPMS has significantly smaller number of clonally expanded Vβs than RRMS ($p=0.05$). Boxes represent values from the 25th to the 75th percentiles, inner lines represent the median, and whiskers show the minimal and maximal values.

that of the difference between NMO and MS. To determine which possibility is correct, we next made statistical analysis of the number of clonally expanded Vβs among NMO, RRMS and SPMS (Fig. 3B). Mean numbers of clonally expanded Vβs were 3.2±3.1 in RRMS and 1.3±1.0 in SPMS. Thus, NMO also had a significantly larger number of clonally expanded Vβ genes than RRMS ($p=0.04$). Furthermore, SPMS had significantly smaller number of clonally expanded Vβs than RRMS ($p=0.05$) although SPMS had significantly longer disease durations and severer clinical disability than RRMS.

We also isolated $CD4^+$ and $CD8^+$ T cells from PBL of some NMO and MS patients using magnetic beads and examined CDR3 spectratypes. It was revealed that the profiles of unseparated PBL were similar to those of $CD4^+$ T cells (not shown). Although mean age of NMO was significantly higher than MS and healthy subjects, the number of clonally expanded Vβs did not correlated to age in NMO patients ($r=0.27$, $p=0.48$). These findings suggest that physiological expansion of CD8-positive T cells with aging did not significantly influence the results obtained in the present study.

3.3. Selective activation of TCR Vβ1 and Vβ13 in NMO

To determine whether there are selectively activated TCR Vβs in PBL of NMO patients, we analyzed the frequency of each of the Vβ expansions in 11 NMO and compared with 21 MS and 25 healthy subjects (Fig. 4A). Analysis of the frequency of Vβ expansions revealed that the Vβ1, Vβ13 and Vβ14 genes were significantly and selectively activated in NMO compared with MS. Five of 11 NMO patients (45%) had clonally expanded Vβ1 genes whereas none of the 21 MS patients had clonally expanded Vβ1 gene ($p=0.002$). Six of 11 NMO patients (55%) and 2 of 21 MS patients (9.5%) had Vβ13 gene expansion ($p=0.01$), and 5 of 11 NMO (45%) and 2 of 21 MS (9.5%) had Vβ14 gene expansion ($p=0.03$). In the comparison between NMO and healthy subjects, more Vβ genes were significantly activated in NMO, i.e. Vβ1, Vβ3, Vβ5.1, Vβ13, Vβ14, Vβ16, Vβ21 and Vβ23.

SPMS had a very small number of clonally expanded Vβs in the above-mentioned analyses. Therefore, we re-analyzed 11 NMO patients compared to the samples taken only from 13 RRMS patients in the MS group (Fig. 4B). Consequently, we obtained the same result, indicating the selective and significant activation of Vβ1 and Vβ13 in NMO. Five of 11 NMO patients (45%) had clonally expanded Vβ1 genes, although none of the 13 RRMS patients (0%) had clonally expanded Vβ1 gene ($p=0.01$), and 6 of 11 NMO (55%) and none of 13 MS had Vβ13 gene expansion ($p=0.003$). However, Vβ14 expansion was not statistically significant, i.e. 5 of 11 NMO and 2 of 13 MS had Vβ14 gene expansion ($p=0.18$).

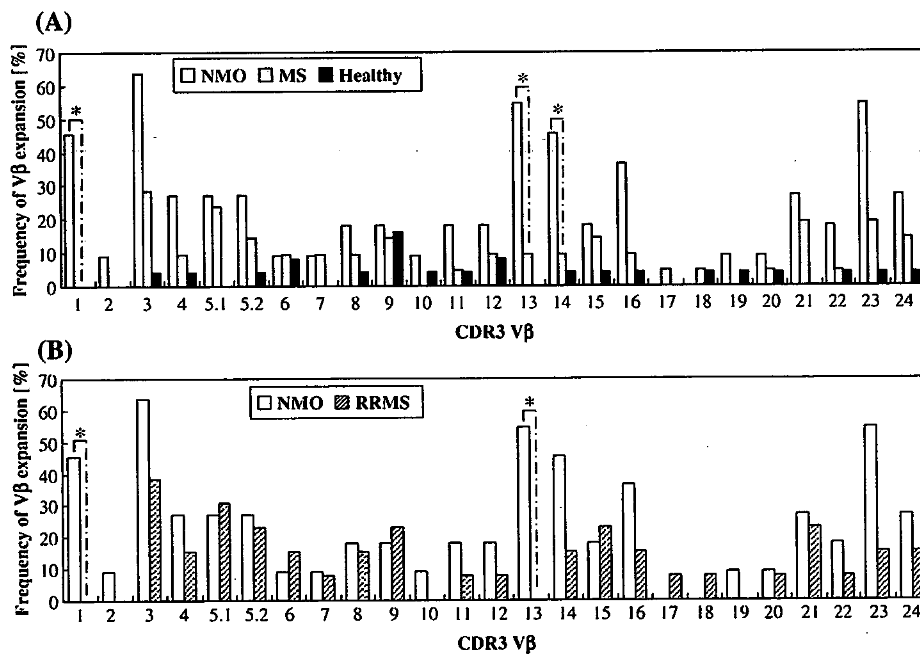

Fig. 4. (A) The frequency of Vβ expansions in PBL of 11 NMO, 21 MS and 25 healthy subjects. Vβ1, Vβ13 and Vβ14 genes are significantly and selectively activated in NMO compared with MS. Five of 11 NMO patients (45%) has clonally expanded Vβ1 genes, although none of the 21 MS (0%) patients has clonally expanded Vβ1 gene ($p=0.002$). Six of 11 NMO (55%) and 2 of 21 MS (9.5%) has Vβ13 gene expansion ($p=0.01$), and 5 of 11 NMO (45%) and 2 of 21 MS (9.5%) has Vβ14 gene expansion ($p=0.03$). In the comparison between NMO and healthy subjects, more Vβ genes i.e., Vβ1, Vβ3, Vβ5.1, Vβ13, Vβ14, Vβ16, Vβ21 and Vβ23, are activated in NMO. (B) The frequency of Vβ expansions in PBL of 11 NMO and 13 RRMS patients. NMO also has selective and significant activations of Vβ1 and Vβ13 than RRMS. Five of 11 NMO (45%) patients has clonally expanded Vβ1 genes, although none of the 13 RRMS patients had clonally expanded Vβ1 gene ($p=0.01$), and 6 of 11 NMO (55%) and none of 13 MS has Vβ13 gene expansion ($p=0.003$). However, Vβ14 expansion is not significantly different between NMO and MS ($p=0.18$).

3.4. Longitudinal changes of the TCR repertoire: TCR activation was correlated with disability of NMO and RRMS

We first made the longitudinal examinations of the TCR repertoire of 11 healthy subjects and found that there was no remarkable change in the number of clonally expanded Vβs in each subjects during the observation period (Fig. 5A). There was no correlation between the number of clonally expanded Vβs and the follow-up period in 32 samples ($r=0.09, p=0.62$).

Fig. 5B depicts the longitudinal changes of 14 blood samples obtained from 6 NMO patients. One patient with low EDSS showed no changes in the number of clonally expanded Vβs with slight increase in EDSS (open circles). Three patients revealed no changes in EDSS but gradual increase in the number of clonally expanded Vβs (open squares, closed triangles and closed squares). Two patients showed increase in the number of clonally expanded Vβs accompanied by increase in EDSS (closed circles and open triangles). We examined the correlation between the number of clonally expanded Vβs and EDSS of 14 PBL and found that there was a significant correlation ($r=0.72$, $p=0.003$).

In the longitudinal examinations of 17 blood samples obtained from 6 RRMS patients (Fig. 5C), 2 patients had no changes in the number of clonally expanded Vβs with increase in EDSS (closed diamonds and closed triangles). The other four patients showed increase in the number of clonally expanded Vβs accompanied by increase in EDSS. This longitudinal pattern of RRMS was thought to be very similar to that of NMO but the correlation was not statistically significant ($r=0.40$, $p=0.11$). This may be attributable to low EDSS scores in the RRMS group.

All of the NMO (Fig. 5B) and RRMS patients (Fig. 5C) with EDSS 6.0 or higher presented more than 5 expanded Vβs. In contrast, all the SPMS patients had EDSS 6.0 or higher and the number of clonally expanded Vβs of 16 blood samples from 6 SPMS was 0 to 4 (Fig. 5D). The latter finding showed no remarkable change during the observation period. Statistical analysis revealed no correlation

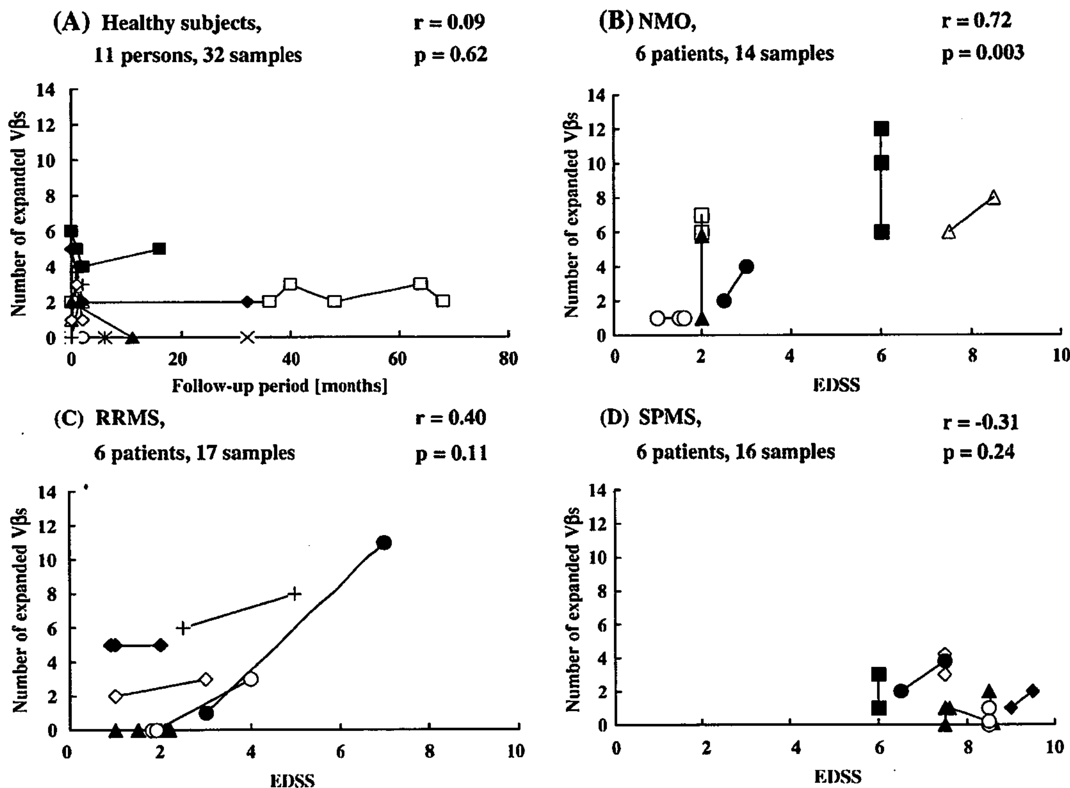

Fig. 5. Longitudinal analysis of the TCR repertoire of 11 healthy subjects (A), 6 NMO, 6 RRMS and 6 SPMS patients. Symbols connected with lines indicate the results of same individuals in multiple examinations. (A) In healthy subjects, there is no remarkable change in the number of clonally expanded Vβs during the observation period. Statistical analysis reveals no correlation between the number of clonally expanded Vβs and the follow-up period ($r=0.09, p=0.62$). (B) Longitudinal changes of the TCR repertoire in NMO. One patient with low EDSS (○) has no changes in the number of clonally expanded Vβs accompanied with slight increase in EDSS. Three patients (▲, □, ■) reveal no changes in EDSS but gradual increase in the number of clonally expanded Vβs. Two patients (●, △) show an increase in the number of clonally expanded Vβs accompanied with increase in EDSS. There is correlation between the number of clonally expanded Vβs and EDSS ($r=0.72, p=0.003$). (C) Longitudinal changes of the TCR repertoire in RRMS. Two patients (◆, ▲) show no changes in the number of clonally expanded Vβs accompanied by increase in EDSS. The rest four patients (◇, ○, +, ●) show increase in the number of clonally expanded Vβs and increase in EDSS. However, it is not statistically significant ($r=0.40, p=0.11$). (D) Longitudinal changes of the TCR repertoire in SPMS. All the SPMS patients show EDSS 6.0 or higher. The number of clonally expanded Vβs of all the patients range from 0 to 4 and there is no remarkable change during the follow-up period. No significant relationship between the number of clonally expanded Vβs and EDSS is noted ($r=-0.31, p=0.24$).

between the number of clonally expanded Vβs and EDSS ($r=-0.31, p=0.24$).

4. Discussion

Elucidation of pathophysiology of NMO is one of the major concerns among Japanese neurologists and neuroimmunologists. While NMO is a relatively rare disease condition in western countries [1–3], this is the major clinical phenotype that accounts for one third of patients with demyelinating diseases in Japan [16,17]. Now, this issue is extending to a worldwide matter of concern and the discovery of a serum autoantibody marker of NMO and Japanese OS-MS (NMO-IgG) had a certain impact [9,18]. In the present study, we showed that there were significant differences in T cell immunity and clonally expanded TCR Vβ genes between NMO and MS. Moreover, it was demonstrated that the longitudinal changes of the TCR repertoire in NMO were similar to RRMS, whereas SPMS patients with longer disease durations and higher EDSS scores consistently had a smaller number of clonally expanded Vβs than RRMS. Although it was previously demonstrated by neuropathology and MRI studies that the progression of SPMS takes place by the non-inflammatory mechanisms [19], it was difficult to detect by blood analysis. To our knowledge, this is the first report showing that the progression of SPMS takes place by the non-inflammatory mechanisms with the longitudinal PBL examinations on living humans.

NMO had an increased TCR Vβ activation and selective clonal expansion of the Vβ1 and Vβ13 genes compared with MS, especially compared with RRMS. This finding suggests that TCR activation in NMO represents the dominancy of cellular immunity in this disease state and was consistent with a previously reported finding that oligoclonal IgG bands in CSF were seldom found in OS-MS/NMO patients [20]. Clonal expansions of selective TCR Vβs including Vβ1 and Vβ13 in NMO suggest that T cells react clonally with autoantigens that induce autoimmune inflammation. Several investigators have reported the association between myelin basic protein (MBP) and TCR Vβ13 [21,22]. Hong et al. demonstrated that Vβ13.1^{+} T cell clones established from MS patients recognized the immunodominant peptide (residues 83–99) of MBP, and those T cells shared an identical CDR3 motif, Vβ13.1-LGRAGLTY. If the TCR usage by pathogenic T cells is known, TCR-based immunotherapy could be achieved in human demyelinating diseases. As reported previously [23,24], encephalitogenic T cells induced in DA rats by immunization with a peptide corresponding to the 62–75 sequence of MBP (MBP_{62-75}) mainly used Vβ10 and Vβ15 and administration of DNA vaccines encoding Vβ10 and Vβ15 successfully ameliorated MBP_{62-75}-induced EAE.

The longitudinal examinations of the TCR repertoire revealed that increase in number of clonally expanded TCR Vβs was correlated with disease deterioration evaluated by EDSS in NMO. Similar tendency was noted in RRMS, but not in SPMS. Therefore, this immunological phenomenon seems to be one of the characteristics of demyelinating diseases with the relapsing-remitting course. At present, we consider that longitudinal increase in clonally expanded Vβs is induced by neuroantigen-specific activation of TCR Vβs as mentioned above. However, the possibility of the bystander activation of T cells by cytokines and chemokines is not fully excluded. In any case, if next relapses are predictable by periodical TCR repertoire examinations, we will be able to start prophylactic therapies during the disease remission.

In the present study, we performed cross-sectional and longitudinal CDR3 spectratyping analysis of PBL taken from Japanese patients with NMO. The results demonstrated that Japanese NMO differs considerably from MS with regard to the nature of the TCR repertoire, but has some resemblance to RRMS with regard to the longitudinal changes of the TCR repertoire. Detailed TCR investigations will provide useful information for the recognition of the clinical and immunological status of NMO and the development of effective antigen-specific and prophylactic immunotherapies.

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