Identification of Autoimmune T Cells Among In Vivo Expanded CD25 $^+$ T Cells in Multiple Sclerosis

The Journal *of* Immunology

This information is current as of September 29, 2022.

Zsolt Illés, Takayuki Kondo, Kazumasa Yokoyama, Takashi Ohashi, Takeshi Tabira and Takashi Yamamura

---

References　This article **cites 22 articles**, 8 of which you can access for free at:
http://www.jimmunol.org/content/162/3/1811.full#ref-list-1

Identification of Autoimmune T Cells Among In Vivo Expanded CD25^{+} T Cells in Multiple Sclerosis[1]

Zsolt Illés, Takayuki Kondo, Kazumasa Yokoyama, Takashi Ohashi, Takeshi Tabira, and Takashi Yamamura[2]

Although clonal expansion of autoimmune T cells has been reported in multiple sclerosis (MS), very limited information is available on specificities, clonal size, or activation state of the expanded clones. Here we address the issue of clonal expansion by using a novel technique demonstrating clonotypes defined by single-strand conformation polymorphism of TCR β-chain cDNAs. Examination of activated T cells (CD3^{+}CD25^{+}) isolated from the peripheral blood of MS revealed limited numbers (20~82) of expanded clones defined by single-strand conformation polymorphism clonotype. To estimate the Ag specificities of dominant clonotypes in the activated T cells, these samples were examined in parallel with Th1 T cell clones specific for myelin basic protein or proteolipid protein (PLP) derived from the same patients. Analysis of two patients demonstrated that the dominant clonotypes would contain those specific for myelin basic protein or PLP. Although the majority of the clonotypes could be detected only transiently, a PLP95–116-specific clonotype was found to persist for over 1 yr. Thus, single-strand conformation polymorphism clonotype analysis allows us to monitor the kinetics of given T cell clones in vivo and could provide useful information for designing clonotype (Id)-specific manipulation of human diseases such as MS. *The Journal of Immunology*, 1999, 162: 1811–1817.

Multiple sclerosis (MS)[3] is a presumed autoimmune disease in which T cells reactive to central nervous system autoantigens such as myelin basic protein (MBP) and proteolipid protein (PLP) may play a central role (Reviewed in Refs. 1 and 2). This postulate is based on substantial evidence, such as increased frequencies of MBP- or PLP-specific T cells in IL-2-stimulated culture of blood T cells from MS vs healthy individuals (3) or significant homologies in TCR CDR3 sequences between MBP- or PLP-specific T cell clones and T cell infiltrates in MS brain lesions (4, 5). Repetitive acquisition of identical T cell clones specific for MBP at different time points also supports the role of MBP-reactive T cells in MS and has been regarded as evidence for persistent expansion of MBP-reactive T cells in MS (6). Furthermore, a PCR-based study measuring TCR mRNAs indicates that the frequencies of MBP-reactive T cells in MS could be much higher than previously estimated (7). These findings suggest the outstanding role of limited numbers of autoimmune T cell clones in the pathogenesis of MS and that specific deletion of these clones by means of T cell vaccination may lead to amelioration of clinical manifestations (8). However, it was not clear in the previous studies (6, 7) whether the T cell clone, estimated to be expanded, was one of a few largest clones in the repertoire or represented the numerous subdominant clones. The advantage of anti-clonotype therapy could be expected only in the former case.

In the present study, we addressed the questions for autoimmune T cell expansion in MS by using a novel technique relying on the single-strand conformation polymorphism (SSCP) of TCR messenger signals. The SSCP-based clonality analysis (9, 10), referred to also as SSCP clonotype analysis, depends on the fact that a single strand of the nucleotide chains would form a unique conformation according to its sequence and migrate to its own position during electrophoresis in the SSCP gel (11). As such, when cDNAs of TCR β-chains from a given sample are amplified by RT-PCR, denatured, and electrophoresed, the TCR message of clonally expanded T cells (clonotype) can be visualized as a distinct band, while those from minor clones are erased in the background smear (Reviewed in Ref. 10). While SSCP clonotype analysis of freshly isolated PBMC or biopsy samples provides basic information for T cell clonality in vivo (9, 10, 12–15), comparison of accumulated T cell clones in different samples is also informative because of the reproducible mobility of each clonotype. As reported in a recent study (16), parallel examination of T cell clones with defined Ag specificities could lead to identification of Ag specificities of accumulated clonotypes in vivo. However, because of limited availability of T cell clones specific for target autoantigens, the feasibility of this strategy has not been formally proven in the field of autoimmune disease research. Owing to a panel of T cell clones specific for putative encephalitogenic epitopes, the present study could provide definitive evidence for expansion and activation of autoimmune T cells reactive to defined epitopes of MBP or PLP in MS. Furthermore, we obtained insights into the dynamics of the autoimmune T cell clones and its relative dominance during clinical course.

Table I. *The list of TCCL used as probes for SSCP clonotype analysis[a]*

Patient	TCCL Code	Specificity	SSCP Clonotypes[b]
IS	4SC5	MBP82–102	3, **6a**, 6b, 6c, 8, 14
	65C6	MBP82–102	**6a**, 6b, **14**
OK	2A7	PLP95–116	5.1, 6, 15
	3C11	PLP95–116	6, 9
	2BA205	PLP95–116	**16**
	2.5	PLP95–116	2a, 2b
	4.1	PLP95–116	14
	4.12	PLP95–116	15
	118.19	PLP118–139	**3**, 6, **8**, **14**, 20
	118.23	PLP118–139	3, **15**, 20
	139	PLP139–155	3, 6, 13a, 13b
SK	95.2	PLP95–116	3, 5.1, 14, 18
	105	PLP105–124	3, 4, 18
	105.18	PLP105–124	1a, 1b, 10, **20**

[a] cDNAs from these TCCLs were used as probes for exploring the possible existence of MBP- or PLP-reactive cells among in vivo expanded clones.

[b] SSCP analysis of cDNAs revealed that each TCCL contains from 1 to 6 clonotypes. Each clonotype is designated by the number of Vβ gene. Independent clonotypes sharing the same Vβ gene are distinguished by alphabetical letter following the Vβ gene number. Clonotypes that were detected in blood or CSF samples are in bold. The β-chain TCR sequences of the in vivo detected clones of TCCL 2BA205, 118.19, 4SC5, and 65C6 were determined, and their amino acid sequences are shown in Table II. The nucleotide sequence data are available from DDDBJ/EMBL/GenBank under accession numbers from AB011247 to AB011254.

Materials and Methods

Subjects

All patients except for one (patient TN with acute MS) had definite MS fulfilling the diagnostic criteria proposed by Poser et al. (17). The diagnosis was further assisted by magnetic resonance imaging in all. Clonal expansion of MBP or PLP peptide-specific T cells was investigated in two relapsing/remitting MS patients (patient OK: 54-yr-old female, DRB1*1501/1502 and patient SK: 45-yr-old male, DRB1*1502/1403) and one secondary progressive MS (patient IS: 30-yr-old female, DR1/DRB1*0410). HLA-DR types were determined by a hybridization protection assay using acridinium-ester-labeled DNA probes (18) or by a standard serological typing. None of the patients received immunosuppressive agents during the period of study.

T cell clones and lines (TCCL)

A panel of CD4^{+} TCRαβ^{+} T cell clones and lines (TCCL) (Table I) had previously been established from PBMC by our modification of the "split-well" technique (19, 20). All the TCCLs (here we use "TCCL" as an operational term representing an independently established long-term culture composed of mono- or oligoclonal T cells) were restricted by HLA-DR in the recognition of MBP or PLP peptide as revealed by proliferation assays with DR-specific blocking Abs or with DR-transfected L cells as APCs (20). They were characterized as Th1-type T cells based on their ability to produce IFN-γ but not IL-4 in response to Ag (our unpublished data). Although there was no particular bias in the use of either MBP82–102, PLP95–116, 105–124, 118–139, or 139–155 peptide for generation of TCCL, our TCCL panel was biased for PLP95–116-specific TCCLs, a majority of which were derived from OK bearing DR2 haplotype. This is consistent with our previous work showing that PLP95–116 is an HLA-DR2-associated epitope in MS (20). SSCP analysis revealed monoclonal or oligoclonal compositions of the TCCLs and characterized the Vβ gene use of each clonotype in TCCL (Table I).

Cell sorting

For analysis of activated T cells, PBMC were doubly stained with anti-CD25-FITC (anti-IL-2 receptor α-chain) and anti-CD3-phycoerythrin (PE) mAbs (Becton Dickinson, Mountain View, CA). The CD3^{+}CD25^{+} fraction was collected by a standard sorting method using a FACSort flow cytometer (Becton Dickinson). CD4^{+} cells were isolated by a magnetic cell sorter MACS (Miltenyi Biotec, Auburn, CA) after labeling with anti-CD4 mAb (PharMingen, San Diego, CA).
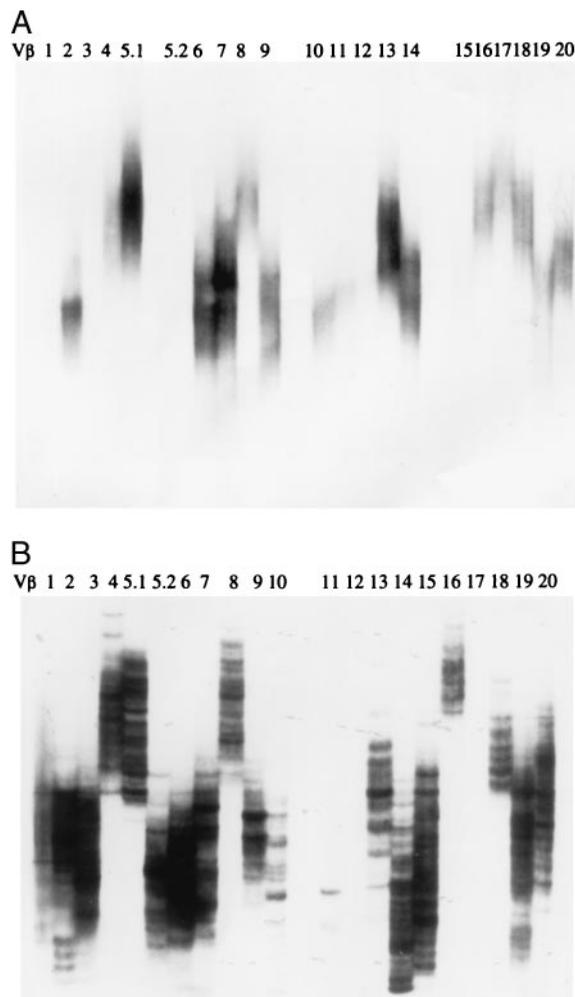

FIGURE 1. SSCP profiles of unseparated PBMC. Unseparated PBMC from healthy subjects (HS) and MS patients were examined by SSCP clonotype analysis. Shown are representative SSCP profiles. Each lane shows an electrophoretic pattern of denatured DNA amplified by a set of a Vβ and the Cβ primers (from left to right: Vβ1–5.1, 5.2, and 6–20). *A*, SSCP profile of an HS showing a smear pattern. *B*, SSCP profile of an MS in relapse demarcating multiple distinctive bands.

SSCP clonotype analysis

SSCP clonotype analysis was performed in essentially the same condition as described by Yamamoto et al. (9, 10). In brief, mRNA was isolated from PBMC, CD3^{+}CD25^{+} cells, cerebrospinal fluid (CSF), or TCCL with QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech, Uppsala, Sweden) and converted to cDNA by using First-Strand cDNA Synthesis Kit (Pharmacia Biotech). One microliter of the diluted cDNA reaction was then individually mixed with a set of a Vβ-specific sense primer and the Cβ antisense primer (30 pmol for each) (21). PCR was performed in 50-μl reactions containing 5 μl of 10× ExTaqBuffer (Takara, Tokyo, Japan), 4 μl of dNTPs, and 2.5 units of ExTaq DNA polymerase (Takara) for 35 cycles (94°C for 30 s, 60°C for 30 s, and 72°C for 1 min), unless otherwise stated. The amplified sample was diluted in a denaturing solution (95% formamide/10 mM EDTA/0.1% bromophenol blue/0.1% xylencyanol) and incubated at 90°C for 2 min for heat denaturation. The sample was then loaded onto a nondenaturing 4% polyacrylamide gel containing 10% glycerol. After electrophoresis, the DNA was transferred to Immobilon-S (Millipore Intertech, Bedford, MA) and hybridized with a biotinylated Cβ-specific sense probe (10). The hybridized anti-sense DNAs were visualized by subsequent incubation with streptavidin, biotinylated alkaline phosphatase, and a chemiluminescent substrate system (Phototope Detection Kit, New England Biolabs, Beverly, MA). To explore Ag specificities of clonotypes found in the blood and CSF samples, the cDNA derived from MBP- or PLP-specific TCCL was run on the adjacent lane as a probe. When we found identical band pairs between the paired samples, electrophoresis was performed at least two more times, varying the sample dilution and/or time

Table II. *TCR amino acid sequences of in vivo expanded clonotypes*[a]

Patient	Specificity	TCCL	Vβ	n-D-n	Jβ	Cβ Gene
IS	MBP82–102	4SC5	Vβ6-LCASSL	NVNSY	NEQFFG-Jβ2.1	2
	MBP82–102	65C6	Vβ6-ND	ND	ND	ND
			Vβ14-LYFCAS	SGLGN	NEQFFG-Jβ2.1	2
OK	PLP95–116	2BA205	Vβ16-YVFCAS	GH	NYGYTF-Jβ1.2	1
	PLP118–139	118.19	Vβ3-YLCAS	TKGH	VLTFG-Jβ2.6	1
			Vβ8-ND	ND	ND	ND
			Vβ14-LYFCAS	LQGAR	YEQFG-Jβ2.7	1

[a] This Table shows the TCR sequences of MBP- or PLP-specific clonotypes whose expansion in vivo were confirmed by the SSCP technique. Each TCCL was run on the gel together with CD25$^+$ T cell population sorted from PBMC or unseparated CSF. Identical bands shared by TCCL and the blood or CSF samples were cut out, and the TCR genes were amplified and sequenced as described in *Materials and Methods*. ND, Not determind. These sequence data are available from DDBJ/EMBL/GenBank under accession numbers from AB011247 to AB011254.

for electrophoresis. Only when the migration positions of the corresponding clonotypes were identical in the additional experiments (80~90% in the cases), the two clonotypes were regarded as being identical.

TCR DNA sequencing

In brief, we cut out a small area of the SSCP gel corresponding to the band and then extracted the TCR clonotype message as previously described (14). The extract was submitted to a second amplification by PCR with a corresponding Vβ (21) and an internal Cβ primer (GCGACCTCGGGT GGGAAC). The PCR products were ligated to M13 mp19 vector arms obtained through *Sma*I digestion. Phages were grown in XL-1 Blue *Escherichia coli* cells (Stratagene, La Jolla, CA), and recombinants were selected by hybridization with a Cβ probe (21). The single-strand DNA was isolated from the positive plaques, and sequencing reactions were induced with BucaBEST Dideoxy Sequencing Kit (Takara) after priming with M13-47 primer.

Results and Discussion

SSCP analysis of unseparated PBMC vs CD3$^+$CD25$^+$ T cells

In preparatory experiments, eleven PBMC samples from healthy subjects (HS) and 21 from MS were examined by SSCP clonotype analysis. In accordance with previous studies (9, 10, 12, 13), the samples from HS showed a smear pattern or demarcated variable numbers of bands on the smear backgrounds (Fig. 1*A*). While samples from MS in remission were not significantly different from HS samples, MS in relapse tended to demarcate higher numbers of bands than HS or MS in remission (Fig. 1*B*). However, there were remarkable individual differences (data not shown), and the SSCP profile in a given subject correlated poorly with the clinical state. Then we examined three PBMC samples from patient OK and two

PBMC from patient SK in parallel with all the PLP-reactive TCCLs derived from the patients shown in Tables I and II. This preliminary analysis showed the presence of a Vβ14 clonotype of TCCL 118.19 in one of the three samples from patient OK and a Vβ20 clonotype in TCCL 105.18 in one of the two samples from patient SK (data not shown), indicating that Ag specificities of clonally expanded T cells could be determined by this method. However, seeing that distinct clonotypes could hardly be demonstrated in samples obtained from MS in remission, we speculated
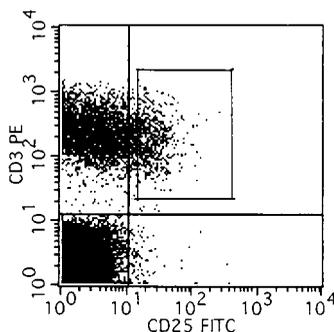

FIGURE 2. Flow cytometric identification of CD3$^+$CD25$^+$ population. PBMC from HS and MS were stained with anti-CD25-FITC and anti-CD3-PE and analyzed by flow cytometer. This is a representative profile of the sample of an MS. The CD3$^+$CD25$^+$ population was sorted and used for SSCP clonotype analysis.
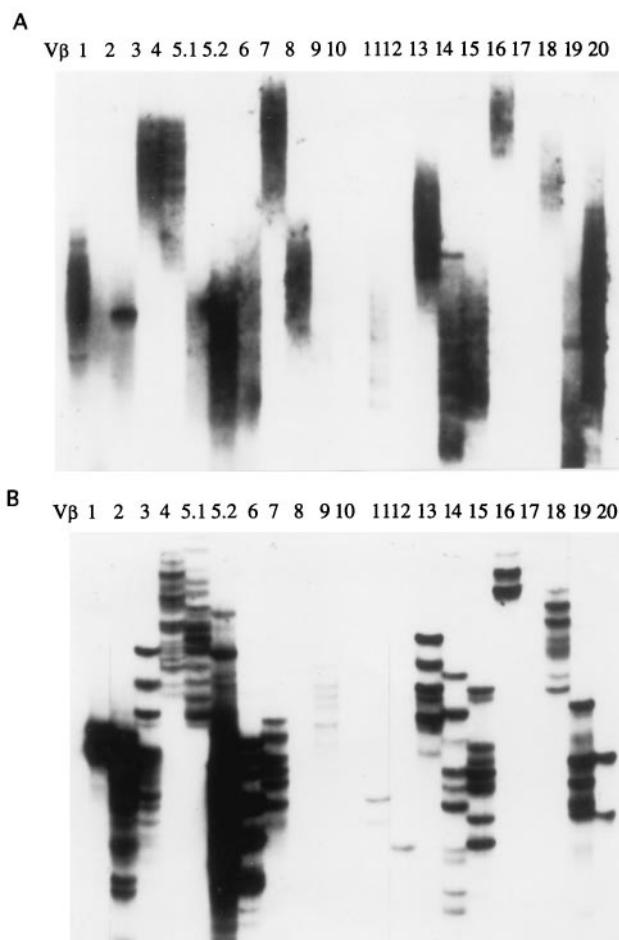

FIGURE 3. SSCP-based comparison of CD3$^+$CD25$^+$ population with unseparated PBMC. The unseparated PBMC of an HS (*upper panel*) was compared with the CD3$^+$CD25$^+$ sorted from the same PBMC (*lower panel*) by SSCP clonotype analysis.

Table III. *Distribution of SSCP clonotypes in the CD3$^+$ CD25$^+$ population*[a]

											Vβ Family												
	1	2	3	4	5.1	5.2	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Total	% of CD25^{+b}
HS																							
HS-1	sm[c]	10	8	6	13	8	6	12	13	10	14	8	3	11	14	14	12	1	6	9	sm	178	8.17
HS-2	4	7	7		3	12	8	3		3	2	2		4	7	2	5		5		2	76	4.14
HS-3	2	12	9	13	15	9	8	7		7		2	1	6	10	11	3		7	7	2	131	1.17
HS-4	7	3	7	4	3	7	6	7	5	4		3	1	5	8	4	9		4	7	8	102	4.46
HS-5	5	sm	5	2	6	10	1	5	10	8	4	6		11	10	11	8		7	9	10	138	4.83
MS																							
IS																							
Jan 97						3	5								6	1	2			3		20	4.05
June 97	3	1	10			2	7	sm							6	8	3		6		3	49	1.87
OK																							
June 96	sm	5	4		8	11	8	7	6	4	8			2		3	5		6		5	82	4.03
July 96	sm	8	5	3	1	sm	3	5		9	10	7	1	1		4	4	5			sm	66	4.07
Nov 96	4	4	6	5	4	5	3	5	4	8		2		6		3	6			5	5	75	3.56
Apr 97	2					2	8	1		2				4	1	13	3		1		8	45	1.62
June 97		1	4	3		8	7			2				2		9	2		4			40	3.35
SA	sm		5	1	6	5	1	8	8	3	6				4	12	7		4	1	2	73	4.41
MS	12	7	9	4	2	11	3	4	9	6	2	4		8	10	6	8		9	8	15	137	5.62
TN	2	2		1								1		6	5			5				22	4.22

[a] The CD3$^+$ CD25$^+$ population was isolated from PBMC of five healthy individuals (HS-1, -2, -3, -4, -5) and five MS patients (IS, OK, SA, MS, TN) by flow cytometry and then processed for SSCP analysis. The number of clonotypes in each Vβ lane and the summation of all bands (total) are shown. Patients IS and OK were repeatedly examined and month/year of the sampling is shown.

[b] The percentage of CD3$^+$ CD25$^+$ T cells among total lymphocyte fraction is shown.

[c] sm, Indicates smear pattern without distinct bands.

that only a small proportion of expanded clones in blood may be visualized by this method. Next we examined the activated T cell population expressing both CD3 and CD25 (IL-2R α-chain) (Fig. 2). Notably, analysis of this population was found to demarcate much higher numbers of distinct clonotypes on the SSCP gels as compared with unseparated PBMC (Fig. 3). Based on this interesting observation, we presume that the mixture of heterogeneous CD25$^-$ population may lower the efficiency in detecting clonal accumulations of CD25$^+$ T cells. Because of technical merits to detect higher number of bands and as activated T cells should play critical roles in vivo, we decided to use the CD3$^+$CD25$^+$ population in following studies.

Limited heterogeneity of in vivo-activated T cells in MS

Collectively, five CD3$^+$CD25$^+$ samples from HS and 10 samples from MS were investigated by the SSCP technique (Table III). The results showed a tendency that the samples from MS demarcate a lower number of clonotypes that are characterized by more limited use of Vβ genes as compared with HS. However, the proportion of CD3$^+$CD25$^+$ cells among total lymphocytes was not significantly different between MS and HS (Table III). An extreme end of the spectrum was seen in the first sample obtained from patient IS with secondary progressive MS, demarcating only ~20 bands with biased TCR usage for Vβ6 and Vβ14 (Fig. 4A) and in patient TN, who has recently developed an acute episode of probable MS. Although less remarkable, samples from relapsing/remitting MS (patient OK) also showed similar profiles characterized by fewer bands and biased Vβ use (Fig. 5, A and B). These results suggest a narrowing of activated T cell repertoire in association with development of MS. Although the underlying mechanism is unclear, we may speculate that T cell recognition targets in MS may tend to be shifted from those for regulatory cells (such as TCR peptides) to a limited epitopes for self-destructive T cells.

The patients' SSCP profiles were not stable during the clinical courses. For example, in the second sample of patient IS (June 1997), the number of SSCP bands had increased up to a total of 50,

and the Vβ usage had become more widely distributed (Fig. 4B). Similarly, the number and distribution of the SSCP bands varied among samples from patient OK (Table III).
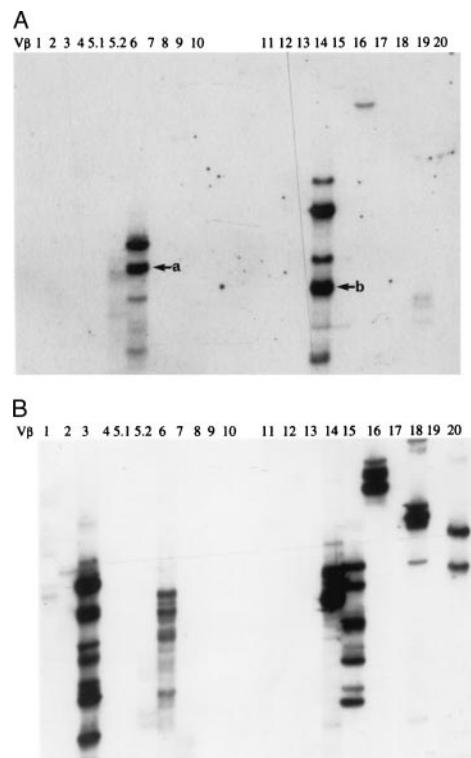

FIGURE 4. SSCP profiles of CD3$^+$CD25$^+$ population from patient IS with secondary progressive MS. *A*, January 1997. Note the reduced number of bands and the marked bias for Vβ6 and Vβ14 usage as compared with HS (Fig. 3). Among these bands, two corresponded to clonotypes of MBP-specific TCCL (arrow a indicates a Vβ6 clonotype of 4SC5, while b corresponds to Vβ14 clonotype of 65C6). *B*, June 1997. Biased Vβ usage has become less remarkable than *A*. See also Table II.
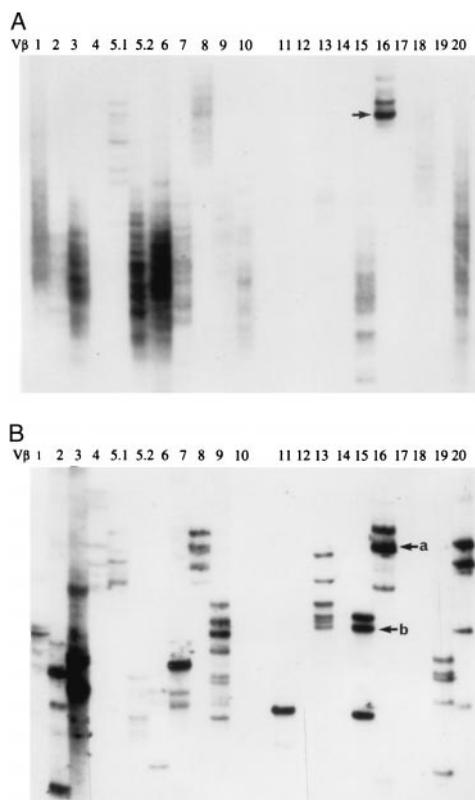
FIGURE 5. SSCP profiles of CD3^{+}CD25^{+} population from patient OK with relapsing/remitting MS. *A*, June 1996. The sample was obtained during a remission phase. The PLP95–116-specific 2BA205 clonotype is indicated by an arrow. *B*, November 1996. The patient was in relapse at the sampling. Arrow a indicates the "persistent" 2BA205 clonotype, and b indicates a clonotype of TCCL 118.23.

Identification of MBP- or PLP-reactive T cells among CD3^{+}CD25^{+} population

We next analyzed 7 of the 10 samples from MS (2 from patient IS and 5 from patient OK) together with the patient-derived TCCLs listed in Table I. The results showed evidence for transient or persistent expansion of MBP- or PLP-specific clonotypes in MS (Table IV and Fig. 6). A most remarkable observation was that two of the dominant clonotypes in the first sample of patient IS (Jan 97) would correspond to those specific for MBP82–102 (indicated by
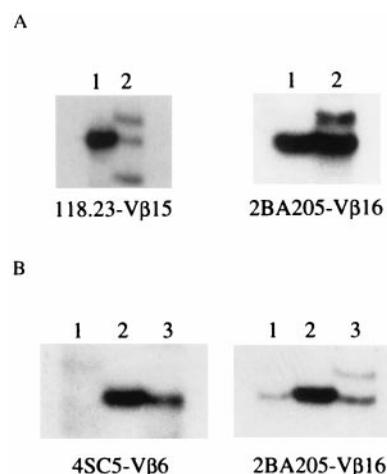

FIGURE 6. Direct comparison of TCCL and in vivo expanded clones. Samples obtained at the same time were directly compared by SSCP analysis. TCCL code and the Vβ gene of examined clonotype are shown below each. *A*, Comparison between TCCL and CD25^{+} T cells. *Lane 1*, TCCL; *lane 2*, CD25^{+} T cells sorted from PBMC. *B*, Comparison of TCCL, CD25^{+} T cells, and CSF. *Lane 1*, CSF; *lane 2*, TCCL; *lane 3*, CD25^{+} T cells sorted from PBMC.

arrow a and b in Fig. 4*A*), implying that a few MBP-specific T cells could play overwhelming roles in certain phases of MS. Together with the fact that the expanded clones are activated in vivo and the corresponding clones belong to Th1 T cells, we speculate that, if a few pathogenic clones can be deleted by anti-clonotype vaccine, it may greatly reduce the clinical activity of this patient in the active phase. It was also striking that a most prominent clonotype in a sample of patient OK corresponded to that of PLP95–116-specific T cell clone 2BA205 (Fig. 5*A*). SSCP analysis and TCR sequencing (Fig. 7) revealed the presence of the 2BA205 clonotype in all the samples, indicating that 2BA205 clone is probably in a continuously activated state in the peripheral circulation (Table IV). Although previous studies suggested the presence of persistent MBP-specific clones (6, 7), it remained elusive whether they were continuously or periodically activated, since the interval of sampling was quite long (1 yr). This is the most convincing proof that myelin-specific T cell clones could exist in a persistently activated state. In contrast, the Vβ15[+] clonotype of 118.23 was found only in a single relapse, and the Vβ8[+] clonotype of 118.19 was found

Table IV. *Temporal profiles for the appearance of autoimmune T cells in Patients IS and OK[a]*

| | | | | MBP82–102 | | | PLP95–116 | PLP118–139 | | | |
| | | | | 4SC5 | 65C6 | | | 118.19 | | | 118.23 |
Patient	Sampling Time	Clinical State	Sample	(Vβ6)	(Vβ6)	(Vβ14)	2BA205 (Vβ16)	(Vβ3)	(Vβ8)	(Vβ14)	(Vβ15)
IS	Jan 1997	Progressive	AT	+	−	+					
	June 1997	Progressive	AT	−	−	−					
	June 1997	Progressive	CSF	+	+	−					
OK	June 1996	Remission	AT				+	−	+	−	−
	July 1996	Remission	AT				+	−	−	−	−
	Nov 1996	Relapse	AT				+	−	−	−	+
	Apr 1997	Relapse	AT				+	−	−	−	−
	June 1997	Intermediate[b]	AT				+	−	−	−	−
	June 1997	Intermediate[b]	CSF				+	+	−	+	−

[a] This table shows the temporal profile for clonal expansion of MBP- or PLP-specific T cells in patients IS and OK. As described in the text, identities of SSCP bands in the collected samples (CD3^{+} CD25^{+}-activated T cells (AT) and CSF) were individually compared with the TCCL listed in Table I. Among 31 clonotypes from the 11 TCCLs examined, eight clonotypes shown in the table were detected at least once in the AT and/or CSF samples.
[b] Intermediate; A recovery state from relapse (not clearly classified as relapse or remission).
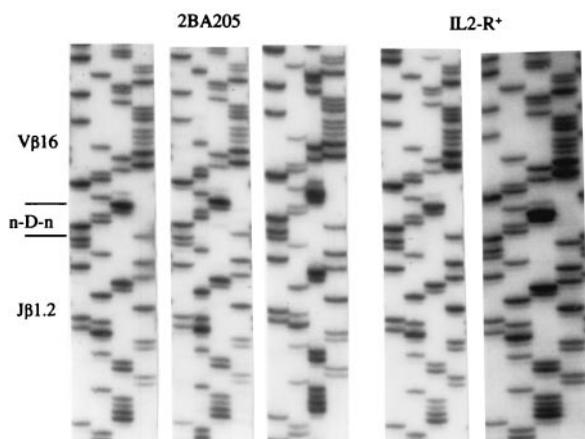
FIGURE 7. Confirmation for TCR sequence identity of corresponding SSCP bands. The TCR sequences of an SSCP band corresponding to the 2BA205 clonotype and of the 2BA205 TCCL were determined as described in *Materials and Methods*. We examined five subclones from the 2BA205 TCCL and five from the corresponding band within the CD3$^+$CD25$^+$ T cells and found that five from the TCCL and four from the corresponding band are identical. Shown are a representative sequencing result: the 2BA205 TCCL (*left panel*); the corresponding band in CD3$^+$CD25$^+$ T cells (*right panel*).

FIGURE 8. Analysis of CSF T cells. After PCR amplification for 38 cycles, the cDNAs derived from CSF were processed and displayed on the SSCP gel. The result of the November 1996 sample from patient OK is shown. Arrow a corresponds to 2BA205, and b and c to clonotypes from TCCL118.19.

only once during remission (Table IV), although these clonotypes are specific for the same PLP epitope. This does not only imply that the kinetics of autoimmune T cell clones are quite heterogeneous but indicates that different mechanisms may be involved for triggering each of these clones with the same epitope specificities. It is tempting to speculate that exogenous superantigens or viral-derived peptides may be involved because they could differentially activate T cells with the same specificity.

Surface phenotypes of the persistent clone

A previous study by Masuko et al. reported that clonal persistence revealed by the SSCP was highly restricted to CD8$^+$ population in healthy individuals who had been infected with a common pathogen (12). In contrast, expansion of CD4$^+$ T cells tended to be transient. Furthermore, since rodent encephalitogenic T cells are CD4$^+$ (22), a theoretical possibility remained that 2BA205 clonotype detected in the CD3$^+$CD25$^+$ population might be expressed by another T cell clone expressing CD8. We therefore separated the PBMC into CD4$^+$/CD4$^-$ fractions by magnetic sorting and examined which of the fractions would contain the TCR message of 2BA205. The results demonstrate that 2BA205 clonotype is specifically found in the CD4$^+$ fraction but not in the CD4$^-$ fraction (not shown). This further supports that the in vivo expanded clone is identical to the PLP-specific 2BA205 clone.

Evidence for presence of autoimmune T cells in CSF

Finally, we analyzed two CSF samples from patients OK and IS. As shown in Table IV, two MBP-specific clonotypes and three PLP-specific clonotypes including 2BA205 were detected in the CSF samples of patients IS and OK, respectively (Fig. 8). Except for 2BA205, the clonotypes detected in the CSF were not found in the CD3$^+$CD25$^+$ T cells obtained at the same time. However, a MBP82–102-specific clonotype of 4SC5 was found in the CD3$^+$CD25$^+$ T cells sampled 5 mo previously, indicating dynamic shift of some autoimmune T cell clones from blood to CSF-central nervous system compartment. These results indicate that the MBP- or PLP-reactive clones could cross the blood-brain barrier and possibly infiltrate the site of inflammation.
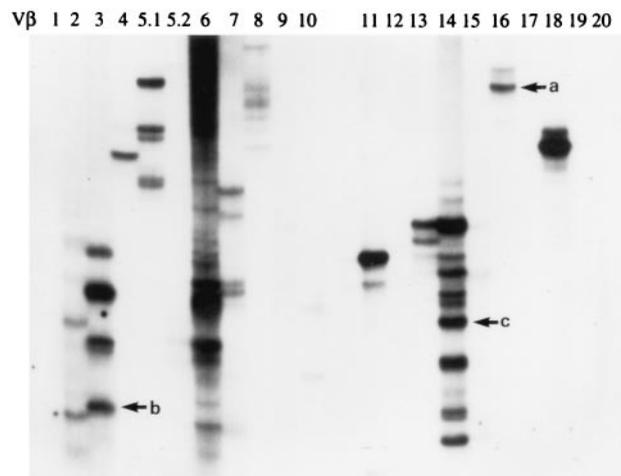
Concluding remarks

Clonal expansion of autoimmune T cells was previously estimated in MS by using limiting dilution analysis (6) or the RT-PCR assay measuring mRNA transcripts encoding TCR chains of MBP-reactive clones (7). These techniques are useful for exquisite analysis of limited populations defined by either Ag specificity (6) or by T cell clonotype (7). However, they do not estimate the significance of examined populations in the total repertoire. In addition, while limiting dilution analysis assays are influenced by multiple factors inherent in in vitro manipulation (7), the PCR assay could handle only a limited number of clones. In contrast, the SSCP analysis (9, 10) figures out the overall profile of dominant clonotypes without depending on in vitro culture. Owing to this technical advantage and the availability of T cell clones specific for putative encephalitogenic peptide, we were able to identify the presence of autoimmune T cells in the activated T cell population of blood and CSF in two patients with MS. Although care should be taken in its use for quantitative estimation, the SSCP analysis is a most reliable and efficient tool for identifying and tracking Ag-specific T cells in human diseases such as MS. Our results revealed that only a small proportion of clonotypes from in vitro established clones can be detected in a series of activated T cell populations in vivo. We assume that the in vivo detected clones might have been involved in the pathology of MS at the time of sampling, while the other clones had played a role previously or may be unrelated to disease as being derived from the naive T cell pool. Such information on in vivo expanded and activated clones might prove truly useful for designing "individually tailored" clonotype-specific treatment for autoimmune diseases in the future.

Acknowledgments

References

1. Hafler, D. A., and H. L. Weiner. 1995. Immunologic mechanisms and therapy in multiple sclerosis. *Immunol. Rev. 144:75.*
2. Martin, R., H. F. McFarland, and D. E. McFarlin. 1992. Immunological aspects of demyelinating diseases. *Annu. Rev. Immunol. 10:153.*

3. Zhang, J., S. Markovic-Plese, B. Lacet, J. Raus, H. L. Weiner, and D. A. Hafler. 1994. Increased frequency of interleukin 2-responsive T cells specific for myelin basic protein and proteolipid protein in peripheral blood and cerebrospinal fluid of patients with multiple sclerosis. *J. Exp. Med. 179:973.*

4. Oksenberg, J. R., M. A. Panzara, A. B. Begovich, D. Mitchell, H. A. Erlich, R. S. Murray, R. Shimonkevitz, J. Sherrit, J. Rothbard, C. C. A. Bernard, and L. Steinman. 1993. Selection for T cell receptor Vβ-Dβ-Jβ gene rearrangements with specificity for a myelin basic protein peptide in brain lesions of multiple sclerosis. *Nature 362:68.*

5. Kondo, T., T. Yamamura, J-i. Inobe, T. Ohashi, K. Takahashi, and T. Tabira. 1996. TCR repertoire to proteolipid protein (PLP) in multiple sclerosis (MS): homologies between PLP-specific T cells and MS-associated T cells in TCR junctional sequences. *Int. Immunol. 8:123.*

6. Wucherpfennig, K. W., J. Zhang, C. Witek, M. Matsui, Y. Modabber, K. Ota, and D. A. Hafler. 1994. Clonal expansion and persistence of human T cells specific for an immunodominant myelin basic protein peptide. *J. Immunol. 152:5581.*

7. Bieganowska, K. D., L. J. Ausubel, Y. Modabber, E. Slovik, W. Messersmith, and D. A. Hafler. 1997. Direct ex vivo analysis of activated, Fas-sensitive autoreactive T cells in human autoimmune disease. *J. Exp. Med. 185:1585.*

8. Zhang, J., R. Medaer, P. Stinissen, D. Hafler, and J. Raus. 1993. MHC-restricted depletion of human myelin basic protein-reactive T cells by T cell vaccination. *Science 261:1451.*

9. Yamamoto, K., H. Sakoda, T. Nakajima, T. Kato, M. Okubo, M. Dohi, Y. Mizushima, K. Ito, and K. Nishioka. 1992. Accumulation of multiple T cell clonotypes in the synovial lesions of patients with rheumatoid arthritis revealed by a novel clonality analysis. *Int. Immunol. 4:1219.*

10. Yamamoto, K., K. Masuko-Hongo, A. Tanaka, M. Kurokawa, T. Hoeger, K. Nishioka, and T. Kato. 1996. Establishment and application of a novel T cell clonality analysis using single-strand conformation polymorphism of T cell receptor messenger signals. *Hum. Immunol. 48:23.*

11. Orita, M., H. Iwahana, H. Kanazawa, K. Hayashi, and T. Sekiya. 1989. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphism. *Proc. Natl. Acad. Sci. USA 86:2766.*

12. Masuko, K., T. Kato, Y. Ikeda, M. Okubo, Y. Mizushima, K. Nishioka, and K. Yamamoto. 1994. Dynamic changes of accumulated T cell clonotypes during antigenic stimulation in vivo and in vitro. *Int. Immunol. 6:1959.*

13. Okubo, M., M. Kurokawa, H. Ohto, T. Nishimaki, K. Nishioka, R. Kasukawa, and K. Yamamoto. 1994. Clonotype analysis of peripheral blood T cells and autoantigen-reactive T cells from patients with mixed connective tissue disease. *J. Immunol. 153:3784.*

14. Yamamoto, K., K. Masuko, S. Takahashi, Y. Ikeda, T. Kato, Y. Mizushima, K. Hayashi, and K. Nishioka. 1995. Accumulation of distinct T cell clonotypes in human solid tumors. *J. Immunol. 154:1801.*

15. Ikeda, Y., K. Masuko, Y. Nakai, T. Kato, T. Hisanuma, S. I. Yoshino, Y. Mizushima, K. Nishioka, and K. Yamamoto. 1996. High frequencies of identical T cell clonotypes in synovial tissues of rheumatoid arthritis patients suggest the occurrence of common antigen-driven immune responses. *Arthritis Rheum. 39:446.*

16. Hoeger, T. A., S. Jacobson, T. Kawanishi, T. Kato, K. Nishioka, and K. Yamamoto. 1997. Accumulation of human T lymphotropic virus (HTLV)-1-specific T cell clones in HTLV-1-associated myelopathy/tropical spastic paraparesis patients. *J. Immunol. 159:2042.*

17. Poser, C. M., D. W. Paty, L. Scheinberg, W. I. MacDonald, F. A. Davis, G. C. Ebers, K. P. Johnson, W. A. Sibley, D. H. Silberberg, and W. W. Tourtellotte. 1983. New diagnostic criteria for multiple sclerosis: guidelines for research protocols. *Ann. Neurol. 13:227.*

18. Matsubara, K., Y. Koide, A. Kobayashi, R. Kaida, S. Takeda, E. Matsuda, Y. Matsuoka, and T. Yoshida. 1992. A rapid and sensitive method for HLA-DRB1 typing by acridinium-ester-labeled DNA probes. *Hum. Immunol. 35: 132.*

19. Inobe, J-i., T. Yamamura, T. Kunishita, and T. Tabira. 1993. T lymphocyte lines and clones selected against synthetic myelin basic protein 82–102 peptide from Japanese multiple sclerosis patients. *J. Neuroimmunol. 46:83.*

20. Ohashi, T., T. Yamamura, J-i. Inobe, T. Kondo, T. Kunishita, and T. Tabira. 1995. Analysis of proteolipid protein (PLP)-specific T cells in multiple sclerosis: identification of PLP95–116 as an HLA-DR2,w15-associated determinant. *Int. Immunol. 7:1771.*

21. Choi, Y., B. Kotzin, L. Herron, J. Callahan, P. Marrack, and J. Kappler. 1989. Interaction of *Staphylococcus aureus* toxin "superantigens" with human T cells. *Proc. Natl. Acad. Sci. USA 86:8941.*

22. Zamvil, S. S., and L. Steinman L. 1990. The T lymphocyte in experimental allergic encephalomyelitis. *Annu. Rev. Immunol. 8:579.*