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Accumulation of Common T Cell Clonotypes in the Salivary Glands of Patients with Human T Lymphotropic Virus Type I-Associated and Idiopathic Sjögren's Syndrome[1]

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To clarify the pathogenesis of human T lymphotropic virus type I (HTLV-I)-associated Sjögren's syndrome (SS), the TCR Vβ gene usage by the infiltrating lymphocytes in the target organ was examined. The Vβ families predominantly used in the labial salivary gland (LSG) from the HTLV-I-seropositive (HTLV-I$^+$) SS patients were more restricted than those from the HTLV-I-seronegative (idiopathic) SS patients, and were commonly Vβ5.2, Vβ6, and Vβ7. The single-strand conformation polymorphism analysis revealed that T cell clonotypes with Vβ5.2, Vβ6, and Vβ7 accumulate in the LSG from the HTLV-I$^+$ and idiopathic SS patients. Among junctional sequences of the most dominant Vβ7 transcripts, the conserved amino acid motif (QDXG: X is any amino acid) was found in six of the five HTLV-I$^+$ SS patients and was also detected in two of the five idiopathic SS patients. Using the probes specific to the motif, the Vβ7 transcripts with the motif were detected in the LSG from all of the seven HTLV-I$^+$ and five of the six idiopathic SS patients, but not from eight healthy subjects. The Vβ7 transcripts with this motif were also detected in the HTLV-I-infected T cell lines obtained from the LSG of an HTLV-I$^+$ SS patient. The accumulation of HTLV-I-infected T cells expressing TCR with the conserved motif was thus indicated. These T cells were commonly present in patients with idiopathic SS and are strongly suggested to most likely be involved in the pathogenesis of both HTLV-I-associated and idiopathic SS. ***The Journal of Immunology,*** **2000, 164: 2823–2831.**

Human T lymphotropic virus type I (HTLV-I)[3] was originally identified to be a causative agent of adult T cell leukemia (1, 2) and is also closely associated with autoimmune-like disorders in various organs, such as HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (3, 4), HTLV-I-associated arthropathy (HAAP) (5), bronchopneumonopathy (6), uveitis (7), polymyositis (8), and mixed connective tissue disease (9). Although numerous studies have been done on the pathogenesis of these HTLV-I-associated disorders, the results have thus far been inconclusive and even conflicting.

Sjögren's syndrome (SS) is considered to be an autoimmune disorder characterized by salivary and lacrimal gland insufficiency resulting in xerostomia and keratoconjunctivitis sicca and has also been implicated to be an HTLV-I-associated disorder (10–13). An association between HTLV-I and SS was first proposed by Vernant et al. (10), on the grounds that HTLV-I-infected patients with HAM/TSP commonly develop SS. Eguchi et al. (11) and Terada et al. (12) later reported a high seroprevalence of HTLV-I among patients with SS in an endemic area for HTLV-I. Terada et al. (12) also suggested that the viral activity is elevated in these patients, especially in the affected lesion. Our recent study also revealed that HTLV-I-associated SS is not essentially different from idiopathic SS and strongly suggested an accumulation of HTLV-I-infected T cells in the salivary glands of patients with HTLV-I-associated SS (13).

Based on these results, we proposed that HTLV-I may be a causative agent of SS and thus formulated the most likely hypothesis for the etiopathologic mechanism of HTLV-I-associated SS (13). To put it very briefly, a self-reactive T cell in a state of anergy could be infected with the virus and activated by the virus, and thereafter could induce this type of autoimmune disorder. Alternatively, the salivary gland environment might possibly allow the preferential accumulation of HTLV-I-infected T cells. In this regard, autoreactivity would be secondary to early degenerative changes in the salivary glands. In this study, to prove this hypothesis, we examined the oligoclonality and specificity of the T cells accumulating in the salivary glands of patients with HTLV-I-associated SS and compared these findings with those from patients with idiopathic SS.

Materials and Methods

Patients

Seven HTLV-I-seropositive (HTLV-I$^+$) and 20 HTLV-I-seronegative (idiopathic) SS patients who were also included in our previous reports (13, 14) and six newly selected idiopathic SS patients were studied. All of the patients were women between the ages of 18 and 71 years who had been

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[3] Abbreviations used in this paper: HTLV-I, human T lymphotropic virus type-I; SS, Sjögren's syndrome; LSG, labial salivary gland; PG, parotid gland; SSCP, single-strand conformation polymorphism; HAM/TSP, HTLV-I-associated myelopathy/tropical spastic paraparesis; HAAP, HTLV-I-associated arthropathy; CDR3, complementarity-determining region 3.

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referred to the Second Department of Oral and Maxillofacial Surgery, Kyushu University Dental Hospital, between 1992 and 1997. All fulfilled the diagnostic criteria for definite SS proposed by the Research Committee on Sjögren's Syndrome of the Ministry of Health and Welfare of the Japanese government (15), and the diagnosis was also based on the diagnostic criteria proposed by the European Community Study Group on Diagnostic Criteria for Sjögren's Syndrome (16). HLA types of the HTLV-I$^+$ SS patients were as follows: patient 1 HLA-A24(9), 26(10); B55(22), 35; Cw1, 3; DR4, 14(6); DQ3, 7(3); patient 2 HLA-A11, 33; B60, 52; Cw7; DR4, 2; patient 3 HLA-A2, 24; B39(16), 61(40); Cw7; DR9, 53; DQ3; patient 4 HLA-A24(19); B54(22), Cw1; DR4, 8; DQ1, 4; patient 5 HLA-A31(19), 24(9); B61(40), 52(5); Cw3; DR2, 11(5); DQ1, 7(3); patient 6 HLA-A24(9); B54(22), 55(22); Cw1; DR4, 53; DQ4; patient 10 HLA-A24(9), 33(19); B52(5), 44(12); DR2, 6. Six of the 7 HTLV-I$^+$ SS patients had other HTLV-I-associated disorders such as HAM/TSP (patients 1, 4, 5, and 6) and HAAP (patients 1, 2, and 3). Of the 20 idiopathic SS patients, 11 patients had primary SS and 9 had secondary SS. Eight healthy subjects without either clinical or laboratory evidence of autoimmune or systemic disease served as controls.

RNA extraction and cDNA synthesis

Heparinized blood samples and labial salivary gland (LSG) biopsies were obtained from all patients, and a parotid gland (PG) biopsy was also obtained from an HTLV-I$^+$ SS patient (patient 10) at the same time. The total RNA was prepared by the acidified guanidinium-phenol-chloroform method as previously described (14, 17, 18). Three micrograms of the total RNA preparation were used for the synthesis of cDNA. Briefly, RNA was incubated for 1 h at 37°C with 20 U of RNasin ribonuclease inhibitor (Promega, Madison, WI), 0.5 μg of oligo(dT)$^{12-18}$ (Pharmacia, Uppsala, Sweden), 0.5 mM of each dNTP (Pharmacia), 10 mM of DTT, and 100 U of RNase H$^-$ reverse transcriptase (Life Technologies, Gaithersburg, MD).

Amplification and semiquantification by PCR

The cDNA were amplified and quantified according to previously described methods (14, 17, 18), with only slight modifications. Briefly, one twenty-third of the TCR β-chain cDNAs were amplified by PCR with the Vβ- and Cβ-specific primers, at a final concentration of 0.4 mM each. The sequences of the specific primers were the same as previously described (14, 18). The amplification was performed with 2.5 U of *Taq* DNA polymerase and a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) under the following conditions: denaturing at 95°C, annealing at 55°C, and extension at 72°C for 1 min each. For a quantitation of the Vβ transcripts, the aliquots of PCR samples were harvested every three cycles after 24-cycle amplification. Those PCR products were electrophoresed through 1.8% agarose gel, transferred to NYTRAN-N (Schleicher & Schuell, Dassel, Germany), and hybridized with the ^{32}P-labeled oligonucleotide Cβ (GTGTTTGAGCCATCAGAA) probe. After hybridization was done for 18 h at 50°C in 1 M sodium chloride, 1% SDS, 10% dextran sulfate, and 100 mg/ml of heat-denatured salmon sperm DNA, the filters were washed twice in 2× SSC with 1% SDS at 50°C. Autoradiographs were generated with the Fuji BAS2000 Bioimage Analyzer (Fuji Photo Film, Tokyo, Japan). After hybridization, the radioactivities of Vβ-specific bands were measured, and the appropriate cycles within an exponential phase of amplification for all the Vβ transcripts were determined. The semiquantitative PCR results were expressed as follows: %Vβ = 100 × (intensity of a Vβ-specific band)/(sum of intensity of all Vβ-specific bands), according to Kerckhove et al. (19).

Single-strand conformation polymorphism (SSCP) analysis

SSCP analysis was performed as described (20). Briefly, amplified DNA was diluted (1:20) in a denaturing solution (95% formamide, 10 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol) and heated to 90°C for 2 min. The diluted samples were then applied to nondenaturing 4% polyacrylamide gels containing 10% glycerol and electrophoresed at 35 W constant power for ~2 h. After electrophoresis, the DNA was transferred to Immobilon-S membrane (Millipore Intertech, Bedford, MA). For visualization of the DNA, the membrane was hybridized with a biotinylated internal Cβ probe (5′-A(AC)AA(GC)GTGTTCCCACCCGAGGTC GCTGTGTT-3′) and detected by subsequent incubation with a streptavidin, biotinylated alkaline phosphatase, and chemiluminescent substrate system (Phototope-Star detection kit; New England Biolab, Beverly, MA). The accumulated clone was counted if a clear band was identified on the background smear, as previously described (20). The total number of clones was then determined to represent the mean value of triplicate independent counts.

Sequencing of cDNAs encoding TCR Vβ genes

A small area of the SSCP membrane corresponding to the position of the distinct band was cut out. The piece was immersed in a Tris-EDTA solution (10 mM Tris-HCl, 0.1 mM EDTA) in a centrifuge microtube and was heated at 80°C for 20 min. The supernatants were then subjected to a second PCR amplification for 36 cycles. Both cloning and sequencing methods were performed as previously described (18). Briefly, the amplified DNA were size-purified on a 1.8% agarose gel and then purified with Microcon-pure (Amicon, Beverly, MA), and then were cloned using a TA cloning kit (Invitrogen, San Diego, CA). After blue/white screening of recombinants on 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) indicator plates, plasmid DNA was extracted from positive colonies and sequenced with a Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems, Foster City, CA) and an autosequencer (Applied Biosystems).

Hybridization with conserved amino acid sequence-specific probes

Biotinylated CASSQDXG probes (5′-TGYGCCAGCAGCCARGAYNN NGGN-3′: R = A + G, Y = T + C, N = A + T + C + G) containing nucleotide sequences for amino acid motif QDXG (X means any amino acid: this motif was conserved in Vβ7 transcripts from SS patients as mentioned bellow) were synthesized. The SSCP membranes were hybridized with the probe for 16 h at 60°C, and probe-specific bands were visualized with the above mentioned detection kit.

Statistical analysis

Results are presented as mean ± SD, and the statistical significance of the differences between the experimental groups was determined by Student's *t* test. *P* values of less than 0.05 were considered to be significant.

Results

TCR Vβ repertoire in the LSG from HTLV-I$^+$ and idiopathic SS patients

An immunohistochemical examination revealed the infiltrates in the LSG from HTLV-I$^+$ SS patients to show a predominance of CD3$^+$ T cells over CD20$^+$ B cells (13). Almost all T cells expressed TCR $\alpha\beta$-chains (data not shown), and this finding was consistent with that in the idiopathic SS patients (14, 17, 21). To examine the oligoclonality of the T cells, we first examined TCR Vβ gene usage in the LSG and PBMC from 7 HTLV-I$^+$ SS patients and compared our findings with those from 20 idiopathic SS patients as previously reported (14). Representative autoradiographs and %Vβ in one patient are shown in Fig. 1, and the results from all patients are summarized in Table I. The Vβ families expressed in the PBMC were diverse in both groups of patients, and the number of detected Vβ families were 17.2 ± 3.4 in the HTLV-I$^+$ SS patients and 18.1 ± 2.6 in the idiopathic SS patients. In contrast, the TCR Vβ repertoire in the LSG was apparently restricted in both groups of patients, and the number of detected Vβ families was 10.4 ± 4.4 in the HTLV-I$^+$ SS patients and 13.3 ± 4.1 in the idiopathic SS patients. The Vβ repertoire in the LSG from the HTLV-I$^+$ SS patients was thus more restricted than in the idiopathic SS patients, but the difference was not statistically significant. As shown in Table I, Vβ5.2, Vβ6, and Vβ7 were more predominant (5, 5, and 7 of the 7 patients, respectively) in the LSG from the HTLV-I$^+$ SS patients. In contrast, in the idiopathic SS patients, the predominantly used Vβ families in the LSG were found to vary in each individual patient and also to differ from patient to patient.

Accumulation of T cell clonotypes in the LSG from the HTLV-I$^+$ and idiopathic SS patients

To confirm the oligoclonality of the T cells, the TCR Vβ clonotypes in the LSG and PBMC were examined by an SSCP analysis, and representative autoradiographs are shown in Fig. 2. In the 20 idiopathic SS patients, most of the PCR products from the PBMC

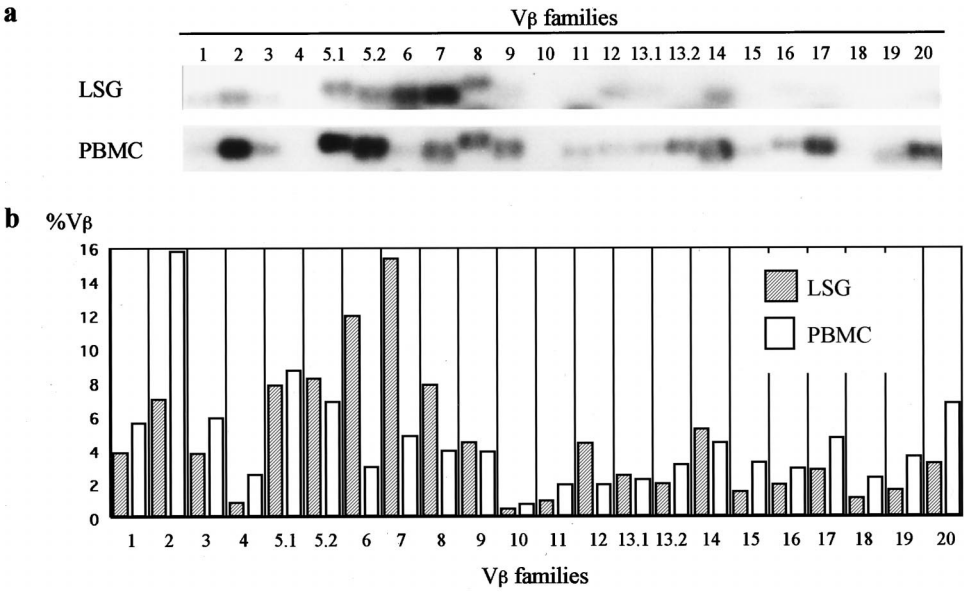

FIGURE 1. The expression of TCR Vβ gene transcripts in LSG and PBMC from an HTLV-I$^+$ patient with SS (patient 6). *a*, Autoradiographs of PCR products for each Vβ family. Vβ transcripts amplified by PCR through 27 cycles were loaded onto 1.8% agarose gel, transferred to a filter, and then hybridized with a ^{32}P-labeled Cβ probe, as outlined in *Materials and Methods*. *b*, The relative amounts of Vβ transcripts in LSG and PBMC are indicated as a percentage, calculated as described in *Materials and Methods*.

developed as a smear, whereas many distinct bands, which indicate accumulating or expanding T cell clonotypes, are detected in those from the LSG. In contrast, many distinct bands were detected in those from both the LSG and PBMC from the 7 HTLV-I$^+$ SS patients. The total number of distinct bands in all Vβ families from the LSG and PBMC was 91.4 $\pm$ 19.6 and 52.8 $\pm$ 4.1 in the 20 idiopathic SS patients, and 71.8 $\pm$ 13.9 and 73.5 $\pm$ 15.5 in the 7 HTLV-I$^+$ SS patients, respectively. Significant differences in the total number of distinct bands in all Vβ families were thus observed between the LSG and PBMC from the idiopathic SS patients ($p < 0.05$) and between the PBMC from the idiopathic and the PBMC from the HTLV-I$^+$ SS patients ($p < 0.05$). Regarding Vβ5.2, Vβ6, and Vβ7, which were predominantly used in the LSG from the HTLV-I$^+$ SS patients, the total number of distinct bands from LSG and PBMC was 19.8 $\pm$ 4.6 and 8.7 $\pm$ 1.6 in the idiopathic SS patients, and 15.3 $\pm$ 2.7 and 16.9 $\pm$ 2.5 in the HTLV-I$^+$

Table I. *TCR Vβ gene usage in the LSG from HTLV-I$^+$ and HTLV-I-seronegative (idiopathic) patients with SS*[a]

Type	Patient[b]	Vβ Families 1	2	3	4	5.1	5.2	6	7	8	9	10	11	12	13.1	13.2	14	15	16	17	18	19	20
HTLV-I$^+$ SS patients	1					↑↑	↑↑	↑	↑↑	↑↑	↑↑			↑			↑↑						
	2						↑↑	↑↑	↑↑														
	3		↑↑			↑↑	↑↑		↑↑		↑					↑							
	4						↑↑	↑↑	↑													↑	
	5						↑	↑↑	↑					↑								↑↑	
	6							↑↑	↑↑	↑↑				↑↑									
	10	↑↑	↑↑	↑↑	↑				↑↑	↑↑				↑								↑↑	
Idiopathic SS patients	1												↑↑						↑↑	↑↑			
	2					↑		↑					↑↑							↑↑			
	3													↑↑			↑						↑
	4		↑↑		↑↑	↑																	
	5							↑↑	↑↑	↑	↑↑			↑↑	↑								
	6									↑↑									↑↑	↑			↑
	7										↑												
	8									↑							↑						↑↑
	9					↑↑	↑		↑	↑					↑								
	10									↑			↑↑							↑			
	11					↑↑	↑↑	↑	↑	↑↑													
	12	↑↑			↑↑		↑																
	13		↑↑				↑																
	14					↑		↑↑	↑↑														
	15					↑			↑↑	↑↑	↑↑												
	16								↑	↑						↑↑	↑↑			↑↑			
	17										↑↑					↑↑							
	18						↑		↑				↑↑			↑↑							
	19		↑↑				↑↑	↑↑	↑↑	↑↑													
	20															↑↑							

[a] The % Vβ of each Vβ family in the LSG was compared with that of the same family in the PBMC. See Materials and Methods for a detailed description of all procedures.
[b] The number of patients is the same as that in previous reports (14, 15).
↑↑, The increase of %Vβ in the LSG was more than 100% when compared with that of the PBMC.
↑, The increase of %Vβ in the LSG was 50% to 100%.
No mark, The increase of %Vβ in the LSG was less than 50%.

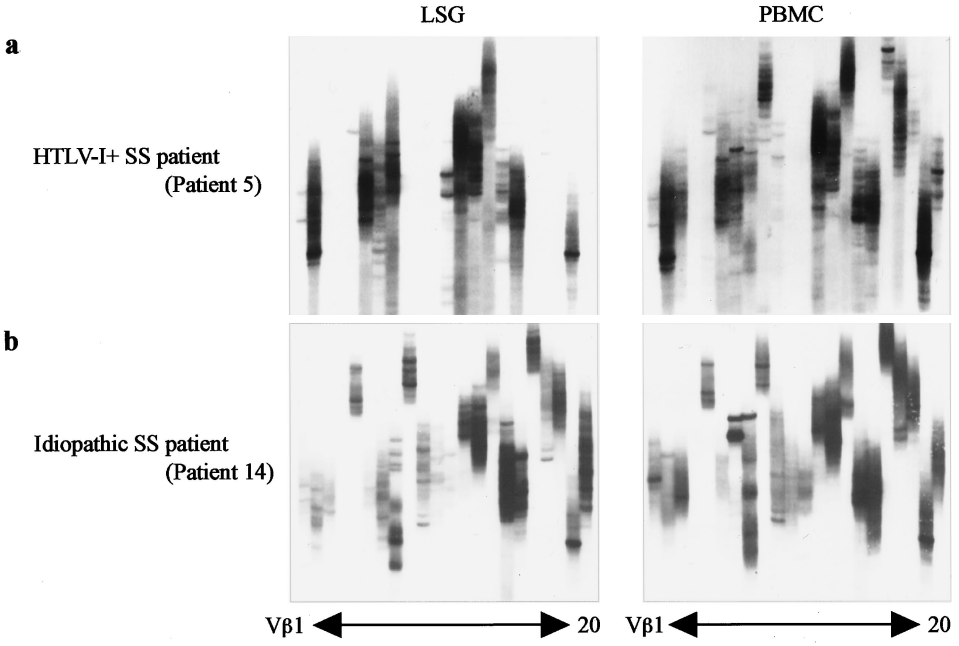

FIGURE 2. An SSCP analysis of TCR Vβ gene transcripts in LSG and PBMC from HTLV-I$^+$ and HTLV-I-seronegative (idiopathic) SS patients. PCR products of Vβ transcripts were denatured into single strands and electrophoresed in a nondenatured polyacrylamide gel by the SSCP method. See *Materials and Methods* for a detailed description of all procedures. *a*, Visualized bands from LSG and PBMC of an HTLV-I$^+$ SS patient (patient 5). *b*, Visualized bands from LSG and PBMC of a idiopathic SS patient (patient 14).

SS patients, respectively. The total number of distinct bands in the 3 Vβ families was significantly different between the LSG and PBMC from the idiopathic SS patients ($p < 0.01$), but not between the LSG and PBMC from the HTLV-I$^+$ SS patients. The total number of distinct bands in the 3 Vβ families in the LSG from the HTLV-I$^+$ SS patients was significantly smaller than that from the idiopathic SS patients ($p < 0.05$), whereas that in the PBMC from the HTLV-I$^+$ SS patients was significantly higher than that from the idiopathic SS patients ($p < 0.01$). The accumulation of T cell clonotypes was thus observed in both the LSG and PBMC in the HTLV-I$^+$ SS patients and only in the LSG in the idiopathic SS patients.

As shown in Fig. 3, especially in Vβ7, which was the most frequently predominant in the LSG from the HTLV-I$^+$ SS patients,

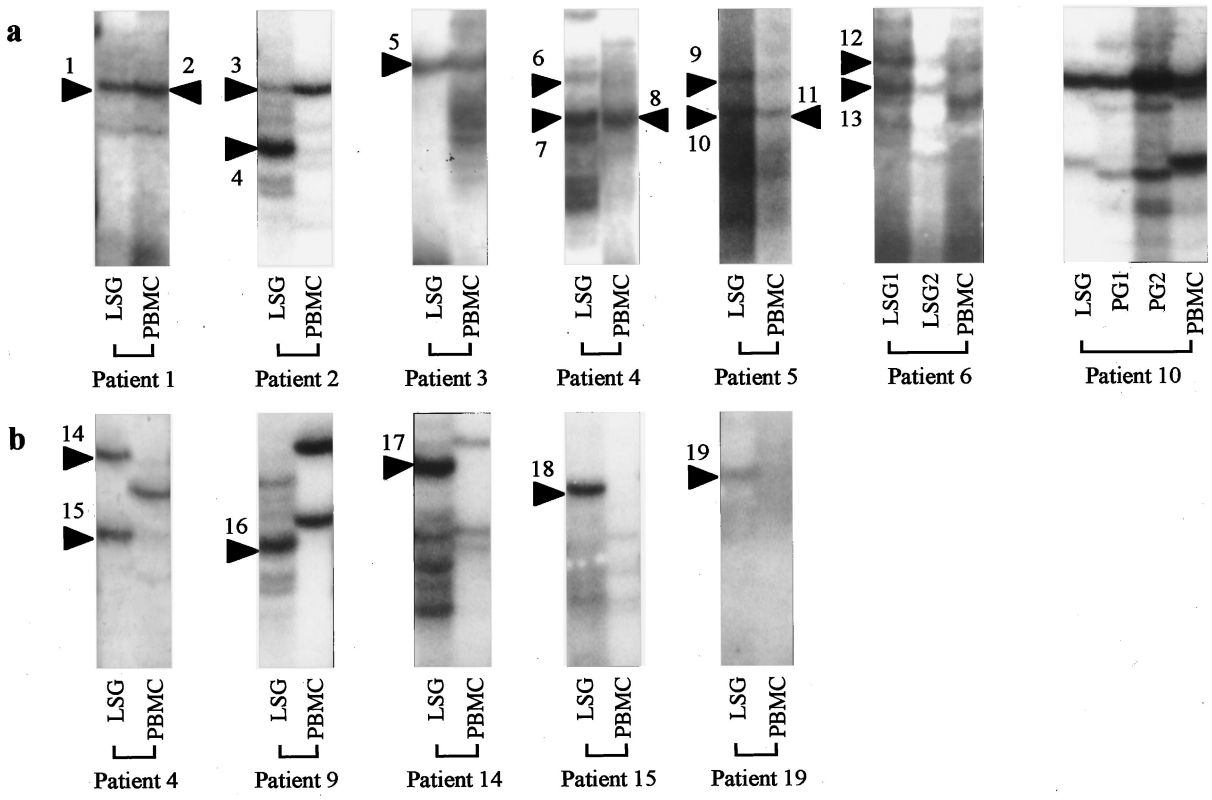

FIGURE 3. Accumulation of T cell clonotypes in Vβ7 transcripts from HTLV-I$^+$ and idiopathic SS patients. PCR products of Vβ7 transcripts were analyzed by the SSCP method as described in Fig. 2. *a*, Visualized bands from LSG, PG, and PBMC from all HTLV-I$^+$ SS patients examined. *b*, Visualized bands from LSG and PBMC from representative idiopathic SS patients. Numbers with closed arrowhead indicate the bands used for sequencing in Table III.

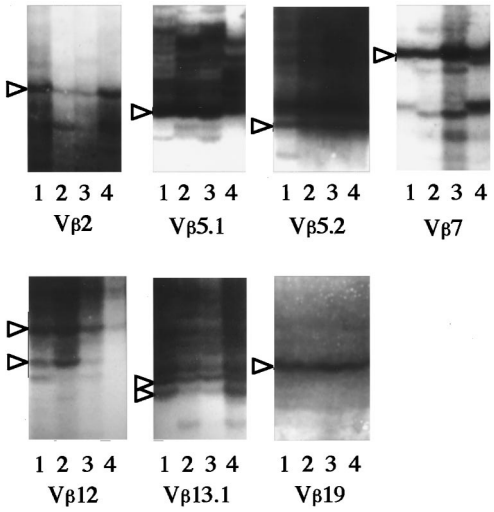

FIGURE 4. Accumulation of T cell clonotypes in multiple Vβ transcripts from different salivary gland tissue specimens from an HTLV-I$^+$ SS patient (patient 10). PCR products of all Vβ transcripts were analyzed by the SSCP method as described in Fig. 2, and results in Vβ families that contain common T cell clonotypes in all samples (open arrowheads) are shown. Lanes: *lane 1*, LSG; *lanes 2* and *3*, two different portions of the same PG; *lane 4*, PBMC.

bands with identical migration patterns were commonly found in the LSG and PBMC from all the HTLV-I$^+$ SS patients. Identical bands were also found in different salivary glands from two individual patients, two different LSG from patient 6, and the LSG and two different portions of the same PG from patient 10. In contrast, in the idiopathic SS patients, distinct bands from the PBMC were less frequently observed than those from the LSG, and no identical bands were found in the LSG and PBMC. Furthermore, in the HTLV-I$^+$ SS patients, identical bands were also found in other Vβ families, although they were less frequently found than those in Vβ7. Autoradiographs from one patient are shown in Fig. 4 since they are considered to be representative.

Complementarity-determining region 3 (CDR3) sequences of the T cell clonotypes in the LSG from the HTLV-I$^+$ and idiopathic SS patients

We next determined the CDR3 sequences of accumulating T cell clonotypes with Vβ7, which develop distinct bands. In the HTLV-I$^+$ SS patients, 10 distinct bands from the LSG and 3 distinct bands from the PBMC were examined (Fig. 3). One or two strongest bands were selected from the LSG of each patient, and the bands from the PBMC were because of their identical migration patterns to those of the bands from the LSG. As shown in Table II, one or more sequences were obtained from one distinct band. Clones with glutamine at position 96 were obtained from all 13 bands, and those with amino acid motifs QDXG and QDXXSY (X means any amino acid) were obtained from 8 and 4 of the 13 bands, respectively. Interestingly, the same amino acid sequence, QDQGP, combined with different Jβ genes, was observed in two patients (patients 2 and 4). Glutamine at position 96 and amino acid motifs QDXG and QDXXSY were thus highly conserved in

Table II. *A CDR3 sequences analysis of TCR Vβ7 clones from SS patients*[a]

Type	Patient[b]	Sample	Band No.[c]	Amino Acid Sequences			Frequency
				Vβ	N-Dβ-N	Jβ	
HTLV-I$^+$ SS patients	1	LSG	1	LCASS	**QD**R**G**QI	YGY (Jβ 1.2)	6/15
				LCASS	TPSEGH	TGE (Jβ 2.2)	6/15
				LCASS	**QD**L**G**A	NYG (Jβ 1.2)	3/15
		PBMC	2	LCASS	**QD**R**G**QI	YGY (Jβ 1.2)	6/10
	2	LSG	3	LCASS	QEPAGTGSY	YGY (Jβ 1.2)	6/12
				LCASS	**QDQGP**	YEQ (Jβ 2.7)	4/12
			4	LCASS	**QD**W**G**LAG	FFG (Jβ 2.1)	5/10
				LCASS	QEGGLDP	QYF (Jβ 2.3)	4/10
	3	LSG	5	LCASS	QDAYRGI	TEA (Jβ 1.1)	10/10
	4	LSG	6	LCASS	**QD**L**G**S	TEA (Jβ 1.1)	6/10
			7	LCASS	**QD**F**TSY**	YGY (Jβ 1.2)	5/12
				LCASS	**QDQGP**	NSP (Jβ 1.6)	4/12
				LCASS	QTGGG	EQY (Jβ 2.7)	3/12
		PBMC	8	LCASS	**QD**F**TSY**	YGY (Jβ 1.2)	8/10
	5	LSG	9	LCASS	**QD**R**GSY**	YGY (Jβ 1.2)	8/10
			10	LCASS	**QD**R**GSY**	YGY (Jβ 1.2)	5/10
				LCASS	QEGQE	NTE (Jβ 1.1)	3/10
		PBMC	11	LCASS	**QD**R**GSY**	YGY (Jβ 1.2)	8/10
	6	LSG	12	LCASS	QMGAGGE	NEQ (Jβ 1.2)	6/12
				LCASS	**QD**Q**G**HD	NEQ (Jβ 2.1)	4/12
			13	LCASS	QMGAGGE	NEQ (Jβ 1.2)	5/10
Idiopathic SS patients	4	LSG	14	LCASS	**QD**R**G**TGE	NIQ (Jβ 2.4)	10/10
			15	LCASS	PSPGD	GYT (Jβ 1.2)	9/10
	9	LSG	16	LCASS	QDGQGV	SGN (Jβ 1.3)	5/10
				LCASS	QYQGD	TEA (Jβ 1.1)	4/10
	14	LSG	17	LCASS	**QD**P**G**TSV	YNE (Jβ 2.1)	5/10
				LCASS	**QD**L**G**G	TEA (Jβ 1.1)	3/10
	15	LSG	18	LCASS	QDVRG	NIQ (Jβ 2.4)	6/10
	19	LSG	19	LCASS	QEGGY	NTE (Jβ 1.1)	5/10

[a] Junctional sequences of TCR Vβ clones obtained from the LSG and PBMC of SS patients were determined as described in *Materials and Methods*, and the sequences that were obtained from more than two clones are listed. The single letter amino acid codes of the 3′ position of TCR Vβ element, the N-Dβ-N (CDR3), and the 5′ position of the J element are given. The bold letters and underlined letters represent conserved amino acid motifs and identical amino acid sequences, respectively.

[b] The number of patients is the same as that in Table I.

[c] The band numbers are the same as those in Fig. 3.

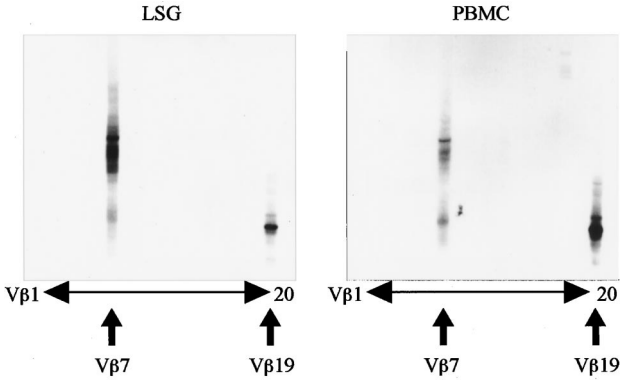

FIGURE 5. The detection of the T cell clonotypes with a conserved amino acid motif in other Vβ transcripts from an HTLV-I$^+$ SS patient. The SSCP membranes of PCR products of all Vβ transcripts were prepared from the LSG and PBMC of an SS patient (patient 5) and were hybridized with biotinylated probes specific to the conserved amino acid motif. See *Materials and Methods* for a detailed description of all procedures.

the accumulating T cell clonotypes with Vβ7. It is also interesting to note that bands with identical migration patterns in the LSG and PBMC from individual patients always contained clones with the same CDR3 sequences.

In the idiopathic SS patients, six distinct bands from the LSG were examined (Fig. 3 and Table II). Glutamine at position 96 and the QDXG motif were also observed in five and two of the six distinct bands, respectively. Regarding the PCR products from the PBMC that develop smeared bands, a total of 40 clones were randomly obtained and examined (data not shown). As a result, glutamine at position 96 and the QDXG motif were observed in only 9 and 6 of the 40 clones, respectively. As a result, glutamine at position 96 and the QDXG motif were commonly conserved in the LSG from both the HTLV-I$^+$ and idiopathic SS patients.

Table III. *Detection of T cell clonotypes with the conserved amino acid motif in Vβ7 transcripts*[a]

Patient	Sample	Frequency (%)
HTLV-I$^+$ SS patients	LSG	7/7 (100%)
	PBMC	7/7 (100%)
Idiopathic SS patients	LSG	5/6 (83%)
	PBMC	3/6 (50%)
Healthy subjects	LSG	0/8 (0%)
	PBMC	0/8 (0%)

[a] The detection of T cell clonotypes with the conserved amino acid motif in Vβ7 transcripts was performed as described in Fig. 6. The frequencies of patients with at least one detectable distinct band are shown.

Detection of the conserved amino acid motif in TCR Vβ genes from SS patients

To examine the presence of T cell clonotypes with the QDXG motif in other Vβ families, the SSCP membranes from the HTLV-I$^+$ SS patients were hybridized with biotinylated CASSQDXG probes (Fig. 5). The distinct bands in the Vβ7 transcript CDR3 sequences that contained the QDXG motif (Fig. 3 and Table II) were all detected with these probes. As a result, distinct bands were detected not only in Vβ7 transcripts from all of the seven HTLV-I$^+$ patients, but also in Vβ19 transcripts from three of the HTLV-I$^+$ patients. As a result, the QDXG motif was not necessarily specific to Vβ7.

To confirm the disease specificity of the QDXG motif, the presence of T cell clonotypes with the QDXG motif was examined in Vβ7 transcripts from newly selected six idiopathic SS patients and eight healthy subjects. As shown in Fig. 6 and Table III, the accumulation of T cell clonotypes with the QDXG motif in the LSG was detected in all of the seven HTLV-I$^+$ SS patients (100%) and five of the six idiopathic SS patients (83%). The accumulation of T cell clonotypes with the QDXG motif in the PBMC was in all of the HTLV-I$^+$ SS patients (100%) and three of the idiopathic SS

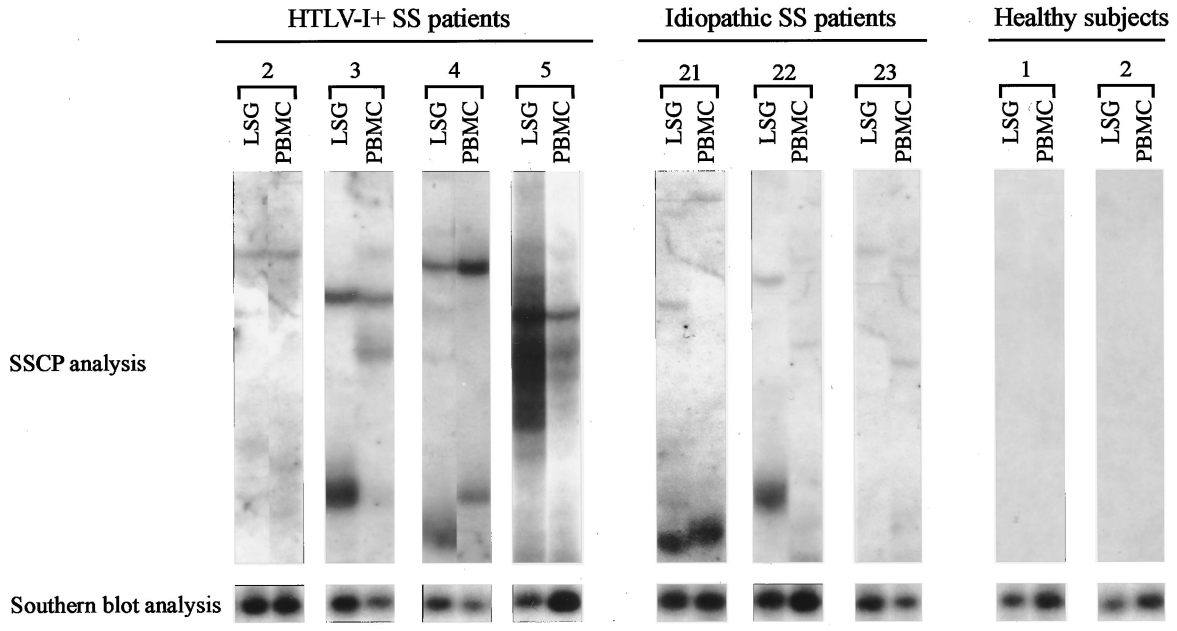

FIGURE 6. The detection of T cell clonotypes with the conserved amino acid motif in the Vβ7 transcripts from HTLV-I$^+$ and idiopathic SS patients. SSCP membranes of PCR products of Vβ7 transcripts were prepared from the LSG and PBMC of SS patients and healthy subjects and were hybridized with biotinylated probes specific to the conserved amino acid motif as described in Fig. 5. Representative visible bands are shown. The PCR products were also electrophoresed through 1.8% agarose gel, transferred to a filter, and then hybridized with biotinylated probes specific to the conserved amino acid motif (Southern blot analysis).

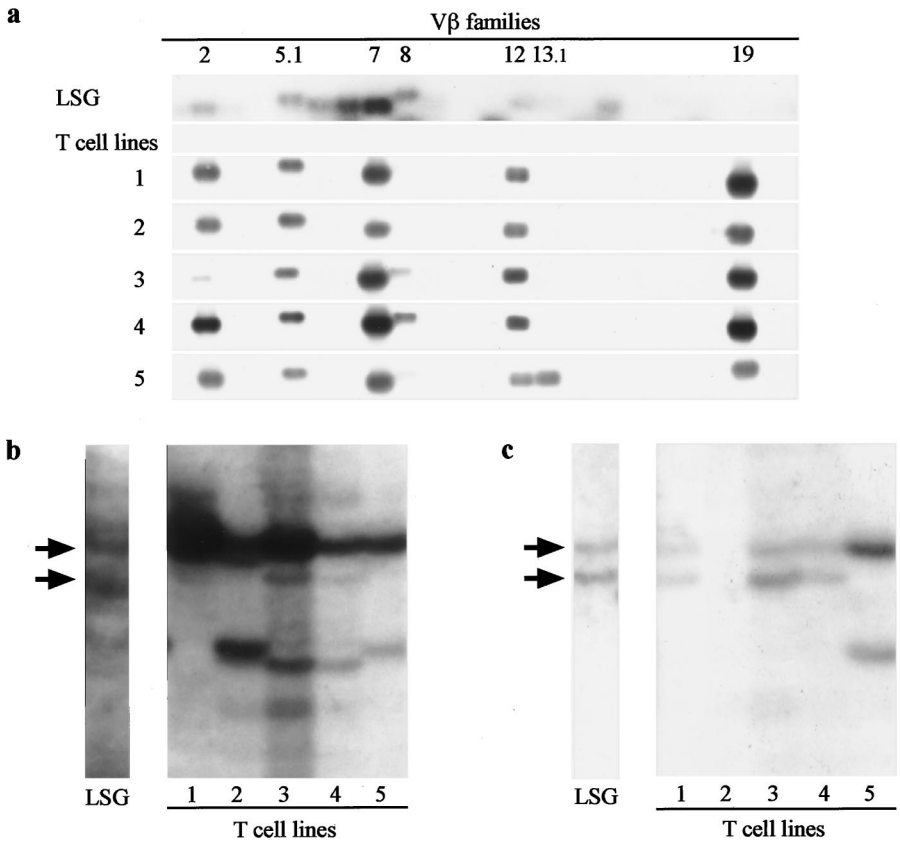

FIGURE 7. The detection of T cell clonotypes with the conserved amino acid motif in the HTLV-I-infected T cell lines obtained from an HTLV-I$^+$ SS patient. Five HTLV-I-infected T cell lines were obtained from the LSG of an HTLV-I$^+$ SS patient (patient 6) as previously reported (13). *a*, PCR products of all Vβ transcripts from these cell lines and the LSG of the same patient were electrophoresed through 1.8% agarose gel, transferred to a filter, and then hybridized with a ^{32}P-labeled Cβ probe, as outlined in *Materials and Methods*. *b* and *c*, The SSCP membranes of PCR products of Vβ7 transcripts were prepared and hybridized with biotinylated Cβ probe (*b*) or biotinylated probes specific to the conserved amino acid motif (*c*), as described in Fig. 5.

patients (50%). In contrast, no distinct band was detected in either the LSG and PBMC from the healthy subjects. These results suggest that T cell clonotypes with the QDXG motif are disease-specifically accumulated in SS patients.

Detection of the conserved amino acid motif in TCR Vβ genes used by T cell lines from the LSG of an HTLV-I$^+$ SS patient

Five HTLV-I-infected T cell lines were obtained from the LSG of an HTLV-I$^+$ SS patient (patient 6) as previously reported (13), and the TCR Vβ gene usage was examined. As shown in Fig. 7*a*, the T cell lines showed similar TCR Vβ repertoire to that of the LSG from the same patient. All of the T cell lines dominantly expressed Vβ7 transcripts, and the %Vβ were 24.0, 19.0, 35.6, 30.6, and 27.4, respectively. On SSCP analysis, the Vβ7 transcripts showed several distinct bands with Cβ probe (Fig. 7*b*), and the major bands contained the transcripts with the QDXG motif (Fig. 7*c*). Furthermore, the transcripts from the four T cell lines contained one or two distinct bands with the QDXG motif, in which the migration patterns were identical to those from the LSG (Fig. 7*c*), and these clonotypes shared identical CDR3 sequences (data not shown). As a result, the HTLV-I-infected T cell clonotypes accumulating in the LSG dominantly express Vβ7 with the QDXG motif.

Discussion

As mentioned in the introduction, we herein attempted to formulate the most likely hypothesis to explain the pathogenesis of HTLV-I-associated SS on the basis of previous reports (10–13). In this study, to prove our hypothesis, TCR Vβ gene usage by the T cells accumulating in the LSG was first examined and compared between HTLV-I-associated and idiopathic SS. The Vβ families predominantly used in the LSG were apparently restricted in HTLV-I-associated SS, whereas Vβ5.2, Vβ6, and Vβ7 were often predominant in the LSG of HTLV-I-associated SS. An SSCP analysis revealed the T cell clonotypes with Vβ5.2, Vβ6, and Vβ7 to accumulate in the LSG in both HTLV-I-associated and idiopathic SS. These results suggest that T cells expressing Vβ5.2, Vβ6, and Vβ7 are involved in the pathogenesis of both HTLV-I-associated and idiopathic SS. It is interesting to note that a smaller number of T cell clonotypes accumulated in the LSG of HTLV-I-associated SS than that of idiopathic SS. In addition, the identical T cell clonotypes also accumulated in the PBMC of HTLV-I-associated SS. A small population of T cells is thus suggested to expand in the periphery before the activation in target organs and then are involved in the pathogenesis of HTLV-I-associated SS.

In HTLV-I-associated SS, highly conserved amino acid glutamine at position 96 and amino acid motifs QDXG and QDXXSY were found in CDR3 of Vβ7 clones from the accumulating T cell clonotypes. The CDR3 interacts with a peptide presented by the MHC molecule (22). Therefore, the presence of a conserved amino acid or amino acid motif in CDR3 suggests that the accumulating T cells recognize common epitopes on Ags. In HTLV-I$^+$ patients, T cells against HTLV-I expand oligoclonally, and the several T cell clones have been reported (23–25). However, none of them had the same CDR3 sequence as those identified in this study. Interestingly, the QDXG motif was also found in Vβ7 clones from the LSG of idiopathic SS. To examine the presence of the QDXG motif both more easily and more extensively, the QDXG-specific probes were prepared and hybridized the SSCP membranes. As a result, the QDXG motif was detected in the LSG from SS patients, except for only one idiopathic SS patient, but not from any healthy subjects. In addition, the QDXG motif was also detected in the PBMC from SS patients (all of HTLV-I$^+$ patients and half of

idiopathic patients), but not from any healthy subjects. These results strongly suggest that T cells expressing TCR with the QDXG motif were specifically present in patients with SS and were also involved in the pathogenesis of SS. To confirm this, an Ag, a candidate self Ag, recognized by T cells expressing TCR with the QDXG motif must be identified, and, to achieve this end, we are presently trying to establish a T cell clone from the HTLV-I-infected T cell lines obtained in this study.

Finally, the TCR Vβ gene usage was examined in HTLV-I-infected T cell lines that were obtained from one HTLV-I$^+$ SS patient. As a result, four of the T cell lines dominantly expressed Vβ7 transcripts with the QDXG motif, which contained identical T cell clonotypes to those from the original LSG tissue. The HTLV-I viral loads of the T cell lines were relatively high, and no T cell line could be obtained from idiopathic SS patients under the same culture conditions (13). Although the HTLV-I-infected T cell lines were still polyclonal, these results strongly suggest that the majority of T cell clones were infected with HTLV-I. The HTLV-I-infected T cells accumulating in the LSG were thus suggested to express Vβ7 gene with the QDXG motif. To confirm these results, we need to examine more HTLV-I-infected T cell lines, as well as T cell clones if possible, from several more HTLV-I$^+$ SS patients.

Taken together, the present study supports our hypothesis that HTLV-I-infected autoreactive T cells are involved in the pathogenesis of HTLV-I-associated SS, although their autoreactivities remain yet to be demonstrated. In HTLV-I carriers, T cells in the periphery are randomly infected. If the virus happens to infect a self-reactive T cell in a state of anergy or ignorance, these cells might first be activated by the virus, and after their tolerance is broken, and then be stimulated by an Ag in an appropriate target organ. These processes may explain the involvement of highly restricted T cell populations, and the clonal expansion of these T cells in the periphery is also suggested in this study. As a result of these processes, HTLV-I-associated autoimmune disorders might be induced in these carriers.

Several studies on HTLV-I infection and HTLV-I-associated disorders may support our hypothesis. A clonal expansion of HTLV-I-infected T cells in the periphery may be supported by reports that an HTLV-I infection is closely associated with an increased number of activated T cells in the PBMC and an increased spontaneous proliferation of the PBMC (26, 27). Furthermore, to explain these findings, Franchini (28) reported the HTLV-I *tax* protein to induce HTLV-I-infected T cells to proliferate independent of cytokines. Scholz et al. (29) also revealed that HTLV-I-infected T cells proliferated in response to antigenic stimulation in the absence of CD80 costimulation. Interestingly, the patients with HTLV-I-associated SS have been reported to show an increased spontaneous proliferation of the PBMC, compared with healthy HTLV-I carriers or patients with idiopathic SS (11). Patients with HAM/TSP have also been reported to show an increased spontaneous proliferation of PBMC and the higher levels of HTLV-I proviral DNA in the PBMC, in comparison with the healthy carriers (12, 30). The accumulation of HTLV-I-infected autoreactive T cells may also be suggested based on the reported findings of other HTLV-I-associated disorders. Our previous study on HAM/TSP (31) showed T cells in the spinal cord to express conserved CDR3 motifs in TCR Vβ genes, which are also found in cases of multiple sclerosis (32, 33) and experimental allergic encephalomyelitis (34, 35). In our report on HAAP (36), the oligoclonal proliferation of HTLV-I-infected T cells in synovial fluid was suggested. In HTLV-I uveitis, Sagawa et al. (37) demonstrated the accumulation of HTLV-I-infected T cells in ophthalmic lesions and thus proposed the presence of activated autoreactive T cells that recognize certain retinal Ags.

Several studies on the TCR Vβ gene usage in idiopathic SS have so far been reported. Sumida et al. (38, 39) initially found a predominant usage of Vβ2 and Vβ13 junctional sequences that showed conserved amino acids at position 106 in CDR3. Matsumoto et al. (40) further reported that T cell clonotypes with an identical TCR Vβ gene commonly accumulated in the lacrimal glands and LSG. In contrast, our previous study (14) revealed that the predominant Vβ gene in the LSG differed both from patient to patient and also within each individual patients. Mizushima et al. (41) also reported that TCR Vβ gene usage in the lacrimal glands is heterogeneous and there are no identical T cell clones between the lacrimal glands and LSG in individual SS patients. The results thus far remain inconclusive and even conflicting, and no disease-specific TCR Vβ gene has yet been identified. Our present study on HTLV-I-associated SS revealed that the oligoclonal HTLV-I-infected T cells accumulating in the LSG have a unique CDR3 motif, and this motif is also present in the LSG of idiopathic SS. As mentioned above, in HTLV-I-associated SS, a smaller number of T cell clonotypes were observed in the LSG than in idiopathic SS. A small number of T cells might thus be sufficient to induce HTLV-I-associated SS, because HTLV-I infection induces T cells to proliferate before activation in an appropriate target organ and thus induce SS more easily. For the same reason, the establishment of a pathogenic T cell clone might also be easier in HTLV-I-associated SS than in idiopathic SS. To understand the pathologic mechanisms of SS, it might thus be worthwhile to study patients with HTLV-I-associated SS.

Recently, several studies have suggested the involvement of retroviruses in the pathogenesis of SS. In patients with AIDS, the glandular destruction commonly observed in SS has been recognized (42). The presence of novel human retroviruses has also been indicated in patients with idiopathic SS (43–46). Furthermore, experimental animals, such as transgenic mice expressing HTLV-I *tax* protein (47) or mice injected with murine leukemia virus (48), have demonstrated exocrinopathy similar to SS in humans. Such findings may suggest that exogenous retroviruses are somehow involved in the pathogenesis of SS. Interestingly, Mariette et al. (49) and Sumida et al. (50) reported the sequences homologous with the HTLV-I *tax* gene, in which the origin remains unknown, to be expressed in patients with idiopathic SS. These findings thus suggest that products encoding the HTLV-I *tax* gene might be candidates for self Ag or could lead to the activation of autoreactive T cells in patients with idiopathic or HTLV-I-associated SS. Further studies elucidating the relationship between HTLV-I infection and SS are expected to provide valuable new insight into the understanding of the pathologic mechanisms of autoimmune disorders.

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