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Diverse T cell receptor β gene usage by infiltrating T cells in the lacrimal glands of Sjögren's syndrome

N. MIZUSHIMA*†, H. KOHSAKA†, K. TSUBOTA‡, I. SAITO† & N. MIYASAKA*† *First Department of Internal Medicine and †Division of Immunological Diseases, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, and ‡Departmentof Ophthalmology, Tokyo Dental College, Chiba, Japan

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SUMMARY

Sjögren's syndrome (SS) is an autoimmune disease characterized by T cell infiltration into the salivary and lacrimal glands (LG). Previous studies on T cell receptor (TCR) usage in the minor salivary glands (SG) have yielded controversial results. We studied TCR β gene usage of the T cells infiltrating to LG, which is the other major target organ of SS. Total RNA was extracted from fresh LG and SG biopsy samples, and peripheral blood mononuclear cells from five SS patients, and converted to cDNA. TCR Vβ gene repertoire was then assessed with quantitative polymerase chain reaction (PCR) assay. Oligoclonality was studied by sequencing V-D-J junctional regions of the PCR products. The TCR Vβ gene usage in LG was diverse in every patient irrespective of disease duration, and similar to that of peripheral lymphocytes from a corresponding patient. The junctional region sequences of over-expressed Vβ families in LG T cells were heterogeneous. We did not find any identical clones shared by LG, SG and peripheral blood. These results showed that the infiltrating T cells in LG of SS patients are polyclonal, and LG and SG do not share the same dominant T cell clonotypes. These suggest that TCR-targeted disease manipulation may have a limited effect on SS.

Keywords Sjögren's syndrome T cell receptor lacrimal gland T cell clonality

INTRODUCTION

Sjögren's syndrome (SS) is characterized by lymphocytic infiltration into the salivary and lacrimal glands (LG), which results in destruction of acini and ducts of the glands. This disease is also called autoimmune exocrinopathy, as systemic exocrine glands can be involved. Most of the infiltrating lymphocytes are $CD4^+$ memory type T cells, which have been thought to play a pathogenic role in SS [1].

Analyses of T cell receptor (TCR) variable (V) gene repertoire of the T cells in inflammatory sites of autoimmune diseases have been carried out to discern the nature of the pathogenic autoreactive T cells [2]. Such TCR repertoire studies may provide supporting evidence of (i) antigen-driven T cell recruitment into the inflammatory loci, or (ii) past or concurrent exposure to superantigens.

On stimulation of T lymphocytes by conventional antigens, the antigens bound to MHC molecules are recognized primarily by TCR(V)-diversity (D)-joining (J)-junctional regions. A single conventional antigen normally expands oligoclonal T cells because the frequency of T cells specific to a given antigen is low. On the other hand, reactivity of T cells to superantigens depends on their TCR Vβ regions but not on the junctional regions. Thus a superantigen expands T cells with one or a few TCR Vβ family gene products. If most of the infiltrating T cells are expanded by a few dominant antigenic determinants or superantigens, they may use unique TCR Vβ gene products. In such instances, TCR-targeted disease manipulation could be feasible.

To date, several studies on TCR usage of infiltrating T cells in SS minor salivary glands (SG) have been carried out, with controversial results [3–6]. This may be partly due to the immunological environment of SG, which is exposed to various food and bacterial antigens in the oral cavity. LG, the other major target organ of SS, is located in a relatively clean area and the infiltrating T cells may better reflect the original disease process. No study, however, has been done on TCR usage in LG of SS.

To determine whether the Vβ repertoire of infiltrating T cells in LG of SS is restricted or biased, we performed the quantitative polymerase chain reaction (PCR) assay on TCR

Correspondence: Nobuyuki Miyasaka, First Department of Internal Medicine, Tokyo Medical and Dental University, 1-5-45, Yushima, Bunkyo-ku, Tokyo 113, Japan.

Vβ gene usage of T cells in LG, comparing it with that of peripheral blood mononuclear cells (PBMC). Then, by DNA sequence analysis of V-D-J regions we asked whether the T cells with over-expressed Vβ families are of oligoclonal origins, and whether they are shared among LG and SG. The results indicate that TCR Vβ repertoires are diverse and infiltrating T cells are polyclonal.

PATIENTS AND METHODS

Patients

Five Japanese SS patients studied were followed at the Ichikawa General Hospital of Tokyo Dental College. Four had primary SS and one had SS associated with rheumatoid arthritis (RA). All patients showed diminished tear secretion and salivary flow assessed by Schirmer test and gum test. All had keratoconjunctivitis sicca. Disease duration, HLA-DR types and serological characteristics are shown in Table 1. LG biopsies for all patients, and minor SG biopsy for one patient, SS-3, were performed without complications after informed consent was obtained. The biopsy samples were frozen in OCT compound (Miles, Napierville, IL) until used for pathological examination or RNA extraction. The pathological study revealed that all had a lymphocytic infiltration with a focus score of more than two. All patients fulfilled classification criteria proposed by Fox *et al.* [7].

RNA isolation and cDNA synthesis

Total RNA was extracted from the frozen samples and the unmanipulated PBMC with a modified single-step RNA isolation method [8]. First-strand cDNA was synthesized from 5 μg total RNA in 20 μl reaction mixture with the SuperScript reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD) and oligo(dT) primers (Life Technologies).

Quantitative PCR assay

To perform the quantitative PCR assay, 0·3 μl of cDNA (derived from 75 ng of total RNA) prepared from LG and SG, or 0·01 μl of cDNA (derived from 2·5 ng of total RNA) prepared from PBMC was amplified in 20 μl of 0·5 U Taq polymerase (Boehringer Mannheim, Mannheim, Germany), 200 μM dNTP, 1·5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl pH 8·3 and 20 pmol of Vβ family-specific and Cβ region-specific primers. The primer sequences for Vβ13.1, 13.2 and Vβ18 were published by Wucherpfennig *et al.* [9], and the other Vβ family primers by Choi *et al.* [10]. The amplification consisted of 30 cycles of 94°C for 1 min, 60°C for 2 min and 72°C for 3 min followed by a final extension for 7 min. LG, SG and PBMC were tested in the same PCR experiment. Eight microlitres of the PCR products were subjected to electrophoresis on 2% agarose gel, and transferred to nylon membranes. The membranes were hybridized with Cβ oligonucleotide probe (5′-CCC GAG GTC GCT GTG TTT GAG CCA TCA GAA-3′) labelled with digoxigenin-dUTP (Boehringer Mannheim). After washing, the membranes were incubated with anti-digoxigenin antibodies conjugated with alkaline phosphatase, which catalysed 3-(2′-spiroadeamantane)-4-methoxy-4-(3″-phosphoryloxy)-phenyl-1,2-dioxetane for luminescent reaction

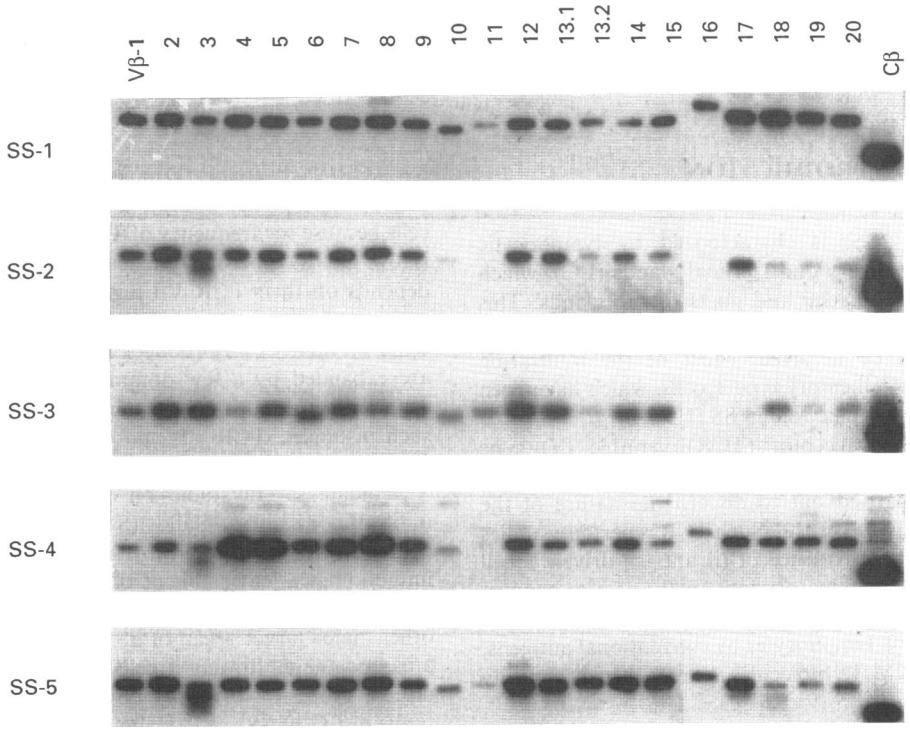

Fig. 1. Expression of TCR Vβ genes (Vβ1–Vβ20) in lacrimal glands (LG) of five Sjögren's syndrome (SS) patients. TCR β-chain gene transcripts were amplified by each Vβ family-specific and Cβ-specific primers. They were gel-electrophoresed and Southern hybridized with the labelled Cβ probe.

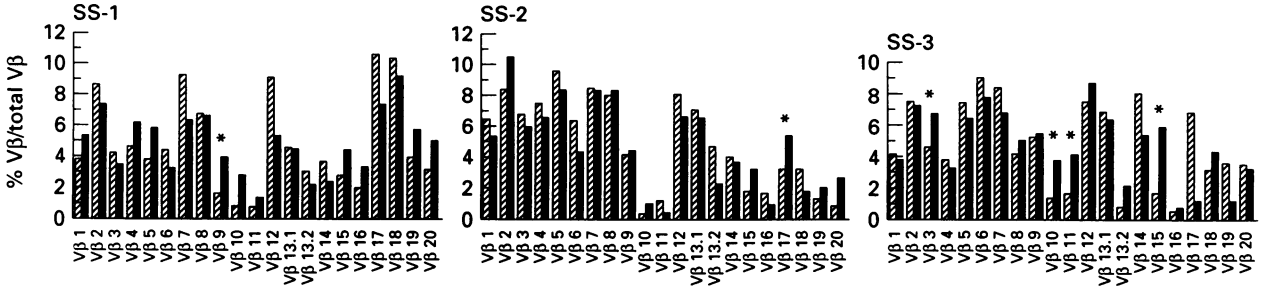

Fig. 2. Expression of TCR Vβ genes in peripheral blood mononuclear cells (PBMC) and lacrimal glands (LG) of SS-1, SS-2 and SS-3. The relative amount of TCR Vβ gene transcripts was described as the ratio (%) of each Vβ to total Vβ. Each Vβ usage was compared between PBMC and LG in each patient. Vβ families marked with asterisks were over-expressed in LG. Vβ9 in SS-1, Vβ17 in SS-2 and Vβ3 and Vβ15 in SS-3 were subjected to DNA sequencing analysis. ▨, PBMC; ■, LG.

(Boehringer Mannheim). The bound probes were detected by placing the membranes under radiographic films for 10–20 min. The intensity of the bands was quantified with a com-puterized laser densitometer (Hoefer Scientific Instruments, San Francisco, CA). The relative amount of TCR Vβ gene expression was calculated as the ratio of the individual Vβ family to the total Vβ band intensity. Reproducibility of the method was confirmed in preliminary experiments, showing that two separate assays of the same cDNA from a healthy donor resulted in almost identical data (mean variation was 0·96%, s.d. was 0·61%). Reliable quantification of the PCR method was substantiated by linear relationship between hybridized band intensity and the amount of initial template cDNA (data not shown). This is essentially based on the method we previously published [11].

Subcloning and sequencing of TCR β cDNA

The amplified TCR fragments were purified from agarose gel. They were directly subcloned into pT7 Blue T-vector (Novagen, Inc., Madison, WI), which were then introduced into *Escherichia coli*, NovaBlue cells (Novagen). Clones with the amplified TCR fragments were screened by digestion with restriction enzyme *Ava* I, which recognizes a sequence in the Cβ region. The inserted TCR genes were sequenced with dye-labelled primers and Ampli Taq DNA polymerase (Applied Biosystems Inc., Foster City, CA) by a 373 A automated DNA sequencing system (Applied Biosystems).

RESULTS

TCR Vβ gene repertoire of LG in SS

The TCR β transcripts were amplified from LG with each Vβ family-specific and Cβ-specific primers. They are gel-electrophoresed and Southern hybridized with the labelled Cβ probe. All investigated Vβ families were expressed in LG of all five SS patients, irrespective of disease duration (Fig. 1). Whereas Vβ1, 2, 3, 4, 5, 6, 7, 8, 12 and 13.1 families were well expressed in almost all patients, Vβ10, 11, 13.2, 16 and 20 families yielded bands with relatively weak intensity in some patients. This may reflect a difference of genetic factors affecting TCR repertoire formation by recombination and thymic selection. In addition, strictly the method employed does not standardize the difference of primer efficiency. However, comparisons of each Vβ expression between different compartments assayed in the same experiment should be valid. We therefore determined the specific change of LG TCR Vβ gene repertoires in SS by comparing Vβ repertoires of LG with those of PBMC from three SS patients (SS-1, SS-2, SS-3) (Fig. 2). For quantification, the intensity of the hybridized band for each Vβ family was scanned with a computerized densitometer. The relative amount of TCR Vβ gene expression was calculated as the ratio of the individual Vβ family to the total Vβ band intensity. Vβ gene usage of PBMC was also heterogeneous. Vβ17 and 18 of SS-1, Vβ2 of SS-2, and Vβ2 and 12 of SS-3, which seemed to be strongly expressed in LG, were also well expressed by PBMC. Therefore, these

Table 1. Characteristics of Sjögren's syndrome (SS) patients analysed

Case	Age	Sex	Primary/ secondary	Duration (years)	HLA	Anti-Ro† (anti-SS-A)
SS-1	53	F	Primary SS	10	DR4,9, DRw53	X 16
SS-2	55	F	Primary SS	2	ND	X 1
SS-3	61	F	Secondary SS*	6	DR4, DRw53	—
SS-4	61	F	Primary SS	5	DR4,2, DRw52,53	X 64
SS-5	68	F	Primary SS	11	DR2	X 40

* SS-3 is a case of secondary SS associated with rheumatoid arthritis.
† All patients are positive for rheumatoid factors and negative for anti-SS-B (anti-La) antibodies.
ND, Not determined.

Table 2. Amino acid sequences of junctional regions of TCR β in Sjögren's syndrome (SS) lacrimal glands

Patient and Vβ family	Frequency	Vβ	NDβN	Jβ	
SS-1					
Vβ9	1/10	YFCASS	PKIWGGA	DTQYFGPGTRLTVL	(JB2.3)
	1/10	YFCAS	IQAGQGD	SPLHFGNGTRLTVT	(JB1.6)
	1/10	YFCASS	PN	DTQYFGPGTRLTVL	(JB2.3)
	1/10	YFCAS	NFRG	YEQYFGPGTRLTVT	(JB2.7)
	1/10	YFCAS	ARAAY	YGYTFGSGTRLTVV	(JB1.2)
	1/10	YFCASS	QGTGD	QPQHFGDGTRLSIL	(JB1.5)
	1/10	YFCASS	QGRTSSC	EQYFGPGTRLTVT	(JB2.7)
	1/10	YFCASS	QRDRGLR	GYTFGSGTRLTVV	(JB1.2)
	1/10	YFCASS			(JB1.4)*
SS-2					
Vβ17	2/10	YLCASS	TGD	SNQPQHFGDGTRLSIL	(JB1.5)
	1/10	YLCAT	ADRGQ	NIQYFGAGTRLSVL	(JB2.4)
	1/10	YLCA	ETGTKRF	YEQYFGPGTRLTVT	(JB2.7)
	1/10	YLCASS	FSGGG	SYNEQFFGPGTRLTVL	(JB2.1)
	1/10	YLCASS	IDWGGM	NEQFFGPGTRLTVL	(JB2.1)
	1/10	YLCASS	RDSSN	NEQFFGPGTRLTVL	(JB2.1)
	1/10	YLCASS	TLTTGD	NEQFFGPGTRLTVL	(JB2.1)
	1/10	YLCASS	TRDSNK	PQHFGDGTRLSIL	(JB1.5)
	1/10	YLCASS			(JB2.7)*
SS-3					
Vβ3	1/12	YLCASS	LNAAY	QPQHFGDGTRLSIV	(JB1.5)
	1/12	YLCASS	FQG	TEAFGQGTRLTVV	(JB1.1)
	1/12	YLCASS	LQD	SPLHFGNGTRLTVL	(JB1.6)
	1/12	YLCASS	SSGTSGR	NEQFFGPGTRLTVL	(JB2.1)
	1/12	YLCASS	LGGR	NIQYFGAGTRLSVL	(JB2.4)
	1/12	YLCASS	FGGVNA	EAFFGQGTRLTVV	(JB1.1)
	1/12	YLCASS	SGQGIL	TGELFFGEGSRLTVL	(JB2.2)
	1/12	YLCAS	QKRGRG	NQPQHFGDGTRLSIL	(JB1.5)
	1/12	YLCAS	LGTG	YEQYFGPGTRLTVT	(JB2.7)
	1/12	YLCAS	KNFG	TEAFGQGTRLTVV	(JB1.1)
	1/12	YLCASS	DGQA	GNTIYFGEGSWLTVV	(JB1.3)
	1/12	YLCASS	LLDARV	TEAFFGQGTRLTVV	(JB1.1)
Vβ15	1/12	YFCATS	DPGQGAS	EQYFGPGTRLTVT	(JB2.7)
	1/12	YFCATS	DPGQQ	SYEQYFGPGTRLTVT	(JB2.7)
	1/12	YFCATS	EGNG	GYTFGSGTRLTVV	(JB1.2)
	1/12	YFCATS	EGTGFG	AFFGQGTRLTVV	(JB1.1)
	1/12	YFCATS	DSE	DTQYFGPGTRLTVL	(JB2.3)
	1/12	YFCATS	GTGG	TDTQYFGPGTRLTVL	(JB2.3)
	1/12	YFCATS	DGN	NQPQHFGDGTRLSIL	(JB1.5)
	1/12	YFCATS	DQRGN	TDTQYFGPGTRLTVL	(JB2.3)
	1/12	YFCAT	REQV	NTEAFFGQGTRLTVV	(JB1.1)
	1/12	YFCATS	DGGQT	YGYTFGSGTRLTVV	(JB1.2)
	1/12	YFCATS	DLGTSS	SYNEQFFRPGTRLTVL	(JB2.1)
	1/12	YFCATS	DGGDRDL	EAFFGQGTRLTVV	(JB1.1)

* Non-productive rearrangement.

changes were not specific to LG, and in general, TCR Vβ repertoire of LG was similar to that of PBMC of the corresponding patient. However, some Vβ families, Vβ9 of SS-1, Vβ17 of SS-2 and Vβ3, Vβ10, Vβ11 and Vβ15 of SS-3 (marked with asterisks in Fig. 2), were over-expressed in LG compared with PBMC (difference of % Vβ expression > mean variation + 2 s.d., as described in Patients and Methods). The over-expressed Vβ families varied depending on the patients, and no over-expression of unique Vβ genes was observed in any of the patients.

TCR β junctional sequences of the amplified TCR from LG of SS patients

We addressed whether there was an oligoclonal T cell expansion in LG, and whether some of the infiltrating T cells in LG have common amino acid motifs.

The size of specific Vβ families can be enlarged by either oligoclonal or polyclonal T cell expansion. Oligoclonal T cell expansion should reflect stimulation by a restricted number of antigens. We selected the Vβ families, Vβ9 of SS-1, Vβ17 of SS-2, and Vβ3 and Vβ15 of SS-3, which were over-expressed in LG compared with PBMC, for clonality analysis. The amplified TCR β fragments including the V-D-J junctional region were subcloned. Ten to 12 cDNA clones from each Vβ family were randomly sequenced. Their deduced amino acid sequences are shown in Table 2. All but two were productive transcripts. None of the Vβ9 clones of SS-1 or Vβ3 and Vβ15 clones of SS-3

Table 3. Amino acid sequences in junctional regions of TCR β of Vβ3$^+$ T cells in salivary glands (SG) and peripheral blood mononuclear cells (PBMC) from SS-3

Tissue	Vβ	NDβN	Jβ	
SG	YLCASS	FWGVNRAGGPI	QFFGPGTRLTVL	(JB2.1)
	YLCASS	SEGWLH	GYTFGSGTRLTVV	(JB1.2)
	YLCASS	LDRDA	GNTIYFGEGSWLTVV	(JB1.3)
	YLCASS	PGTDPF	GELFFGEGSRLTVL	(JB2.2)
	YLCASS	NPGDG	YNEQFFGPGTRLTVL	(JB2.1)
	YLCASS	LA		(no JB)
PBMC	YLCAS	RITGGG	NTEAFFGQGTRLTVV	(JB1.1)
	YLCASS	LGSG	ETQYFGPGTRLLVL	(JB2.5)
	YLCA	YRGRG	NSPLHFGNGTRLTVT	(JB1.6)
	YLCASS	LAGAVG	TQYFGPGTRLTVL	(JB2.3)
	YLCASS	SNVRTGSG	TQYFGPGTRLTVL	(JB2.3)
	YLCASS	LPAT	DTQYFGPGTRLTVL	(JB2.3)
	YLCASS	SSGEG	QETQYFGPGTRLLVL	(JB2.5)
	YLCASS	HEAALAPG	EQFFGPGTRLTVL	(JB2.1)
	YLCASS	NRGAG	ETQYFGPGTRLLVL	(JB2.5)

had the same junctional region sequence. Among the Vβ17 clones in SS-2, only two of 10 clones had identical sequences. However, identical TCR sequences with such frequency were commonly observed in normal peripheral blood [12–15]. These results indicate that the T cells infiltrating to LG were polyclonal.

Some common motifs in amino acid sequences of V-D-J junctional region, such as -Leu(L)Gly(G)- in SS-3 Vβ3 and -Asp(D)Pro(P)Gly(G)- in SS-3 Vβ15 were found. However, since these motifs were observed in PBMC as well (Table 3 for Vβ3, data not shown for Vβ15), they were not unique to LG. Furthermore, there was no significant restriction of Jβ gene usage in the Vβ families investigated. The length of junctional regions was also variable.

TCR β sequences among LG, SG and PBMC

If dominant antigen(s) exist in both LG and SG, and induce T cell infiltration, some of the T cell clonotype should be shared among LG and SG. We studied T cell clonotypes of LG, SG and PBMC of one SS patient, SS-3. TCR Vβ3 transcripts, which are over-expressed in LG, were amplified from SG T cells and PBMC of the same patient. The junctional regions of the TCR β-chain were then sequenced. Table 3 shows the deduced amino acid sequences of six clones from SG and nine from PBMC. As shown in Tables 2 and 3, no shared transcript was found among the LG, SG and PBMC samples. These results showed that LG and SG do not share the same dominant T cell clonotypes.

DISCUSSION

The present study has revealed that TCR Vβ repertoires of LG-infiltrating T cells in SS do not have unique Vβ biases, and are similar to peripheral T cell repertoires. Sequence analysis of TCR β junctional regions of the over-expressed Vβ transcripts showed that the LG-infiltrating T cells are diverse. All these findings indicate that the LG-infiltrating T cells are of polyclonal origins. Furthermore, no identical clone was shared by LG, SG or PBMC.

There are three possible mechanisms underlying the diverse TCR in SS. First, most of the T cells infiltrating in the inflammatory sites might be recruited non-specifically. This is supported by our findings that the Vβ repertoires of LG and PBMC share similar patterns in individual patients. In LG and SG of SS, cell adhesion molecules on vascular endothelial cells, such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), are up-regulated [16,17]. These molecules can readily promote non-specifically activated lymphocytes to adhere to the local vessels and to migrate into the inflamed tissues. In this regard, only < 5% of T cells at the inflammation sites are specific to *Mycobacterium leprae* antigen in tuberculous leprosy [18] and to myelin basic protein (MBP) in experimental autoimmune encephalomyelitis (EAE) [19].

Second, there might be a wide variety of antigenic determinants in LG of SS. During the course of MBP-induced EAE, epitopes on MBP are not fixed, but altered by disclosure of cryptic epitopes [20,21]. Such spreading of antigenic determinants occurred at most within 40 days. In SS, even if the initial T cell response is primed by a single antigen, multiple epitopes could emerge and generate diverse TCR repertoires. SS is a chronic disease, onset of which is not clear. The patients are usually asymptomatic at the early stage. By the time clinical symptoms manifest themselves, TCR repertoires could have already become diverse, as observed in our study.

Third, even T cells specific to a single epitope can have different Vβ family chains [22]. Again in rat EAE induced with MBP, encephalitogenic T cells reactive to a dominant epitope, MBP(68–88) primarily utilized TCR Vβ8.2, whereas T cell lines specific to MBP(86–88) carried diverse TCR Vβ chains [23].

There are conflicting data on the clonality of infiltrating T lymphocytes in minor SG of SS. It has been reported that Vβ2 and Vβ13 family genes are predominantly expressed and their Jβ usage is biased [3,4]. Dwyer *et al.* [5] observed a dominant use of Vβ13 gene in SG of SS, but no significant association with particular Jβ genes. By analysing the Vβ usage of IL-2-stimulated T cells derived from SG of SS, Legras *et al.* [6] reported that the Vβ repertoires were different among the patients, and there was no predominant use of a unique Vβ gene, although they showed 'a clear restriction' of Vβ gene

usage at an especially early stage with grade 1 or 2 lymphocytic infiltration. In our study on T cells in LG, we did not observe any TCR Vβ biases.

The different results could be ascribed to a difference in studied organs, methods and patients. Since SG, especially minor SG, are located in the oral cavity where numerous food and bacterial antigens are present, these antigens could modify the immune reaction specific to SS. On the other hand, the tear contains various bactericidal substances which may create a cleaner environment for LG. The TCR repertoire in LG may therefore better reflect the original immune response specific to SS. *In vitro* stimulation can introduce an artificial bias in the Vβ repertoire. In addition, all the studies of SG used PCR-based assay. When TCR genes are amplified from tissues with a small amount of T cells, TCR repertoire data can sometimes show a restricted use of a few Vβ genes. In our preliminary experiments, we studied two small pieces from a single biopsy sample with a little lymphocytic infiltration, and obtained the two discrepant TCR repertoires, probably due to the paucity of the TCR transcripts present in the samples. The results of tissues with early-stage, scarce lymphocytic infiltration may suffer from this artefact. We therefore used fresh samples containing a large amount of T cells to avoid these serious artefacts.

We have demonstrated that the TCR β usage of infiltrating T cells in LG of SS is diverse. To detect a smaller population of clonally expanded T cells, complementarity determining region 3 (CDR3) length analysis or single-strand conformation polymorphism analysis [24] might be useful methods. Based on our study, the T lymphocytes involved in inflammation of SS are essentially polyclonal, suggesting that TCR-targeted disease manipulation such as anti-Vβ antibody therapy may have a limited effect on SS.

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