Study 1: Analysis of TCR repertoire in normal BALB/c mice

Five mice in one group (four groups: 3 and 7 weeks of age, males and females, total 20 mice) were sacrificed by exsanguination under isoflurane anesthesia, and their spleens, lymph nodes, thymuses, lungs, intestinal tracts, livers, genital organs (uteruses and seminal vesicles), bone marrows, and brains were collected. Total RNA samples were isolated from these tissues using an RNeasy Mini Kit (QIAGEN Sciences, Maryland, USA) according to the manufacturer's instructions.

Real-time PCR

The CD3 mRNA expression levels in the total RNA samples were examined by real-time PCR using a LightCycler apparatus (Roche Diagnostics, Basel, Switzerland). Total RNA (500 ng) was converted to cDNAs using a PrimeScript™ RT Reagent Kit (Takara Bio, Otsu, Japan) according to the manufacturer's instructions. PCR amplification was performed using SYBR® Premix Taq™ (Takara Bio) for SYBR® Green I. A primer pair specific for CD3 (forward primer: CACTCTGGGCTTGCTGATGG; reverse primer: TCATAGTCTGGGTTGGGAACAGG) was purchased from Takara Bio. Briefly, the PCR amplification was carried out in a 20-μl final volume containing 2 μl of DNA template, 0.4 μl of 10 μM solutions of the CD3 forward and reverse primers, 10 μl of SYBR® Premix Taq™ and H_2O up to 20 μl. After an initial denaturation step at 95°C for 10 s, 50 cycles of 95°C for 5 s and 60°C for 30 s were performed, with measurement of the fluorescence at the end of each cycle. Immediately after the amplification, a melting curve analysis was performed from 65°C to 95°C at a linear temperature transition rate of 0.1°C/s with continuous recording of fluorescence. The absolute copy numbers of unknown samples were calculated by comparing the threshold cycles with the corresponding standard curve. A reference cDNA was used in every assay to correct experimental variations among assays. The cDNA levels among samples were normalized by the expression level of the internal control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [25]. Quantification was based on the relative ratio between each unknown sample and the reference sample. To include all measurement mRNAs, the reference sample was mixed with total RNA of various organs and tissues.

AL-PCR

To analyze the TCR repertoires, samples were amplified by AL-PCR as previously described [13, 28]. Briefly, freshly isolated RNA (1 μg) was converted to double-stranded cDNAs using SuperScript™ II RNase H⁻ Reverse Transcriptase (Invitrogen, California, USA) according to the manufacturer's instructions, except that a specific primer (BSL-18E) was used. P10EA/P20EA adaptors were ligated to the 5' ends of the cDNAs and the adaptor-ligated cDNAs were cut with *SphI*. First and second PCR amplifications were performed with TCR α chain constant region (TCRAC)-specific or TCR β chain constant region (TCRBC)-specific primers and P20EA. A third PCR amplification was performed using both P20EA and 5'-biotinylated TCRAC- or TCRBC-specific primers for biotinylation of PCR products. All PCR amplifications were performed in triplicate.

MHAs

The TCR α chain variable region (TCRAV) and TCR β chain variable region (TCRBV) repertoires were analyzed by MHAs [13, 14, 28]. Briefly, 10 pmol of amino-modified oligonucleotides specific for TCRAV and TCRBV segments were immobilized in carboxylate-modified 96-well microplates (C type; Sumitomo Bakelite, Tokyo, Japan) with water-soluble carbodiimide. The probes used in the present study were described previously [28]. The names of the TCR families and probes were designed in order of the genomic TCR gene name, except for the pseudogene. Prehybridization and hybridization were performed in GM Church (GMC) buffer (pH 7.0) comprising 0.5 M Na_2HPO_4, 1 mM EDTA, 7% SDS and 1% BSA at 47°C. Denatured 5'-biotinylated PCR products (40 μl) were mixed with an equivalent volume of 0.4 N NaOH/10 mM EDTA, and added to 4 ml of GMC buffer. An aliquot (100 μl) of the hybridization solution was placed in each well of the microplate containing immobilized oligonucleotide probes specific for the V segment. After hybridization, the wells were washed six times with washing buffer (2 × SSC, 0.1% SDS) at room temperature. For stringent washing, the plate was incubated at 37°C for 10 min. After three washes, 200 μl of TB-TBS buffer (pH 7.4) comprising

10 mM Tris-HCl, 0.5 M NaCl, 0.5% Tween 20, and 0.5% blocking reagent (Roche Diagnostics, Mannheim, Germany) was added to block non-specific binding. Next, 100 μl of alkaline phosphatase-conjugated streptavidin (R&D Systems, Minnesota, USA) diluted 1:5000 in TB-TBS was added, and the plate was incubated at 37°C for 30 min. After six washes in T-TBS (10 mM Tris-HCl pH7.4, 0.5 M NaCl, 0.5% Tween 20), color development was initiated by adding 100 μl of substrate solution, 4 mg/ml p-nitrophenylphosphate (Nacalai Tesque, Kyoto, Japan), to 10% diethanolamine (pH 9.8). After 1 h, the absorbance was determined at 405 nm. Data were analyzed using the Ascent software package (Thermo Fisher Scientific, Massachusetts, USA). The V/C value was determined using TCR cDNA-concentrated samples containing the appropriate V segment and the universal C segment, respectively [28]. The absorbance obtained for each TCRV-specific probe was divided by the corresponding V/C value. The relative frequencies were calculated based on the corrected absorbance using the following formula: relative frequency (%) = [(corrected absorbance of TCRV-specific probe) / (sum of corrected absorbance of TCRV-specific probes)] × 100.

Study 2: Analysis of TCR repertoire in HC treated mice

The HC treatment of mice was performed by a modified Reichert *et al.* method [20]. In brief, a group of five 3-week-old male mice were administered i.p. with 5.0 mg of HC acetate (Wako, Osaka, Japan) dissolved in 200 μl of saline. A separate group of five mice were administered i.p. with 200 μl of saline and served as controls. At 48 h after the administration, all mice were sacrificed by exsanguination under isoflurane anesthesia, and their thymuses and spleens were collected. In these samples, flow cytometry analysis and TCR repertoire analysis (according to the method of total RNA isolation, AL-PCR and MHAs) were carried out.

Flow cytometry analysis

Cell suspensions were prepared in PBS containing 5% BSA (BSA-PBS). Thymus and spleen cells were stained with the following monoclonal antibodies: CyChrome-conjugated American Hamster anti-CD3ε (145-2C11; BD PharMingen, New Jersey, USA); fluorescein isothiocyanate (FITC)-conjugated rat anti-CD4 (H129.19; BD PharMingen); and phycoerythrin (PE)-conjugated rat anti-CD8a (53-6.7; BD PharMingen). After staining, cells were washed with BSA-PBS and analyzed on a FACS-Calibur flow cytometer (Becton Dickinson, California, USA) using the CellQuest software. Cells were gated on forward scatter/ side scatter plots and then on CD3.

Table 1. Comparison of CD3 mRNA expression levels in organs and tissues[a)]

Organs and tissues	Relative expression[b)] (%) ± SD
Spleen	75.8 ± 2.6
Lymph nodes	145.4 ± 8.6
Thymus	82.2 ± 8.5
Lungs	24.5 ± 1.2
Intestinal tract	4.6 ± 0.1
Liver	1.4 ± 0.1
Seminal vesicles	2.0 ± 0.1
Uterus	0.4 ± 0.1
Bone marrow	1.7 ± 0.1
Brain	0.1 ± 0.1

[a)]The assay was performed by five independent experiments. The assay results indicate the ratio of the number of T cells and/or expression intensity of T cell receptors in organs and tissues. [b)]The relative expression (%) represents the percentage value of each sample divided by the reference sample.

Statistical analysis

Differences between groups were analyzed by Student's paired *t*-test using StatView 5.0 for Windows (SAS Institute, North Carolina, USA). *P* values of <0.05 were accepted as statistically significant.

Results

Comparison of CD3 expression in various organs

The CD3 mRNA expression levels were analyzed in the spleen, lymph nodes, the thymus, the lung, the intestinal tract, the liver, the genital organs (uterus and seminal vesicles), the bone marrow, and the brain of normal 3-week-old male BALB/c mice (Table 1). The expression levels of CD3 in the spleen, lymph nodes and the thymus were notably greater than those in the lung and the intestinal tract. Whereas the liver, genital organs (the uterus and the seminal vesicle), the bone marrow, and the brain expressed extremely low levels of CD3 compared to the lung and the intestinal tract. Similar CD3 expression patterns were found in female mice (data not shown).

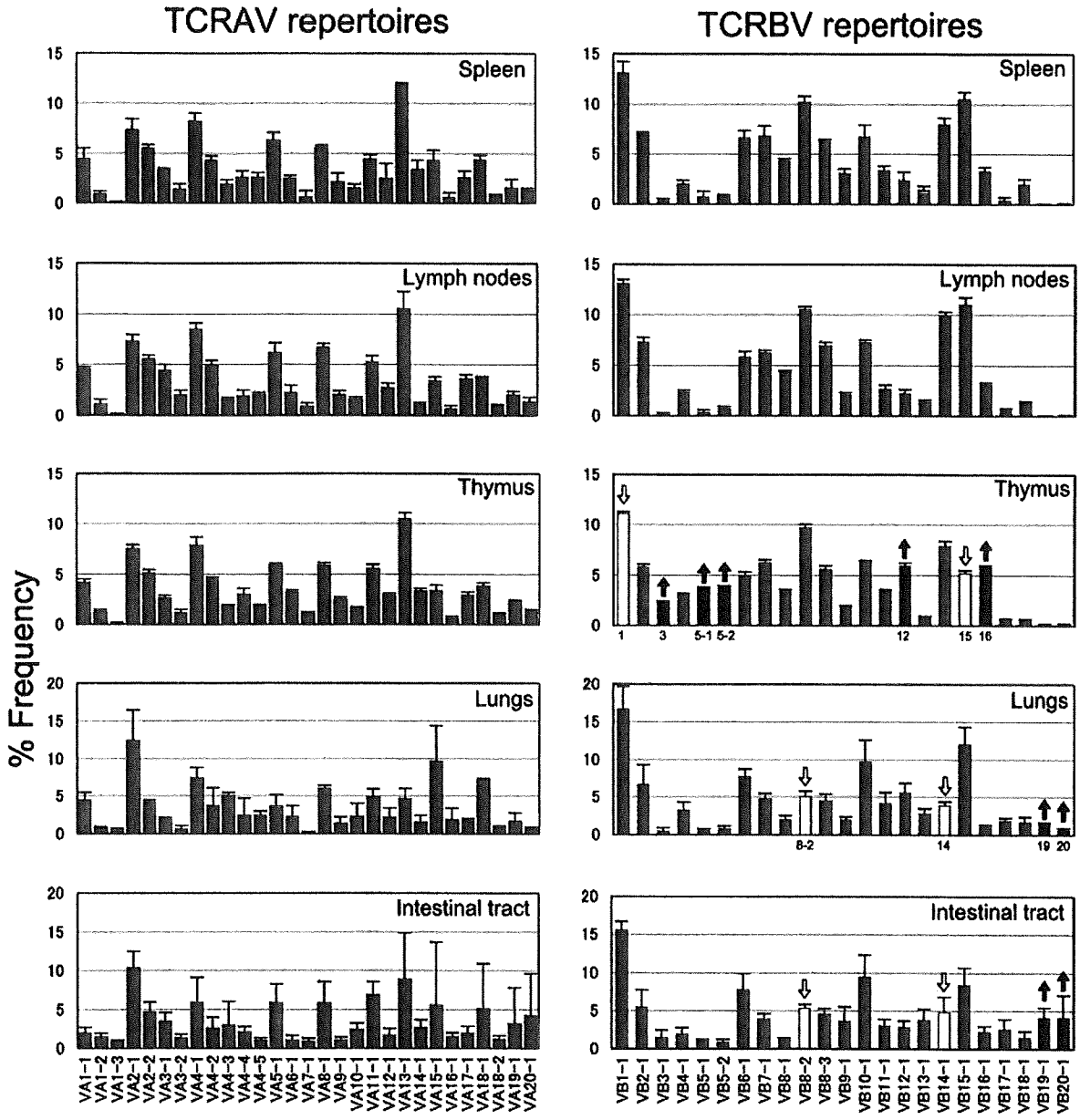

Fig. 1. TCRAV and TCRBV repertoires in tissues of 3-week-old male BALB/c mice. The percentages of cells bearing each TCR are shown for the spleen, lymph nodes, the thymus, the lung, and the intestinal tract. Vertical error bars indicate the standard deviation of five independent experiments. In the thymus, the percentages of cells bearing TCRBV3-1, 5-1, 5-2, 12-1, and 16-1 were significantly increased ($P<0.05$; black arrows and bars) and the percentages of cells bearing TCRBV1-1 and 15-1 were significantly decreased ($P<0.05$; white arrows and bars) compared with the corresponding levels in the spleen. In the lungs and the intestinal tract, percentages of cells bearing TCRBV19-1 and 20-1 were significantly increased ($P<0.05$; black arrows and bars) and the percentages of cells bearing TCRBV8-2 and 14-1 were significantly decreased ($P<0.05$; white arrows and bars) compared with the corresponding levels in the spleen.

Determination of TCRAV and TCRBV repertoires in tissues

The TCR repertoires of 3-week-old male BALB/c mice were analyzed in various organs and representative TCR repertoires are shown in Fig. 1. The TCRAV repertoires were consistent among the spleen, lymph nodes, and thymus, while the TCRBV repertoires were consistent between the spleen and lymph nodes. The TCRBV

repertoires in the thymus differed from those in the spleen and lymph nodes. In the thymus, the percentages of cells bearing TCRBV3-1, 5-1, 5-2, 12-1, and 16-1 were significantly increased and those of cells bearing TCRBV1-1 and 15-1 were significantly decreased in comparison with the spleen and lymph nodes.

The TCRAV and TCRBV repertoires in the lung and intestinal tract differed from those in the spleen, lymph nodes, and the thymus. Smaller numbers of TCRBV8-2- and TCRBV14-1-bearing cells were detected in the lung and the intestinal tract compared to the lymphatic organs. On the other hand, though virtually no TCRBV19-1- and TCRBV20-1-bearing cells were detected in the spleen, lymph nodes, and the thymus, they were apparently present in the intestinal tract and, to a lesser extent, in the lung.

Smaller numbers of subfamilies were detected with weak signals in the liver, seminal vesicles, uterus, bone marrow, and brain and their repertoires differed from those in the spleen, lymph nodes, and the thymus (data not shown).

Comparison of TCR repertoires in mice of different sexes and ages

The TCRBV repertoires were analyzed in the spleen, lymph nodes, and the thymus from 3- and 7-week-old male and female mice (12 groups). The TCRBV repertoires in mice of each sex and age are shown in Fig. 2. The percentages of TCRBV15-1-bearing cells were significantly higher in males than in females in both age groups, but there were no apparent differences in any of the other TCR repertoires. On the other hand, the percentage of TCRBV1-1-bearing cells was significantly decreased in the spleen, lymph nodes, and the thymus in 7-week-old mice compared with 3-week-old mice, whereas the percentages of TCRBV8-3- and 14-1-bearing cells were significantly increased in lymphatic organs of older mice.

Alterations in T cell populations in the thymus and spleen induced by HC administration (Table 2)

The number of thymic T cells decreased from 32.5 × 10^7 to 2.9 × 10^7 after HC administration, indicating the dose adopted in this study was effective. In control mice, approximately 80% of the thymic lymphocytes were

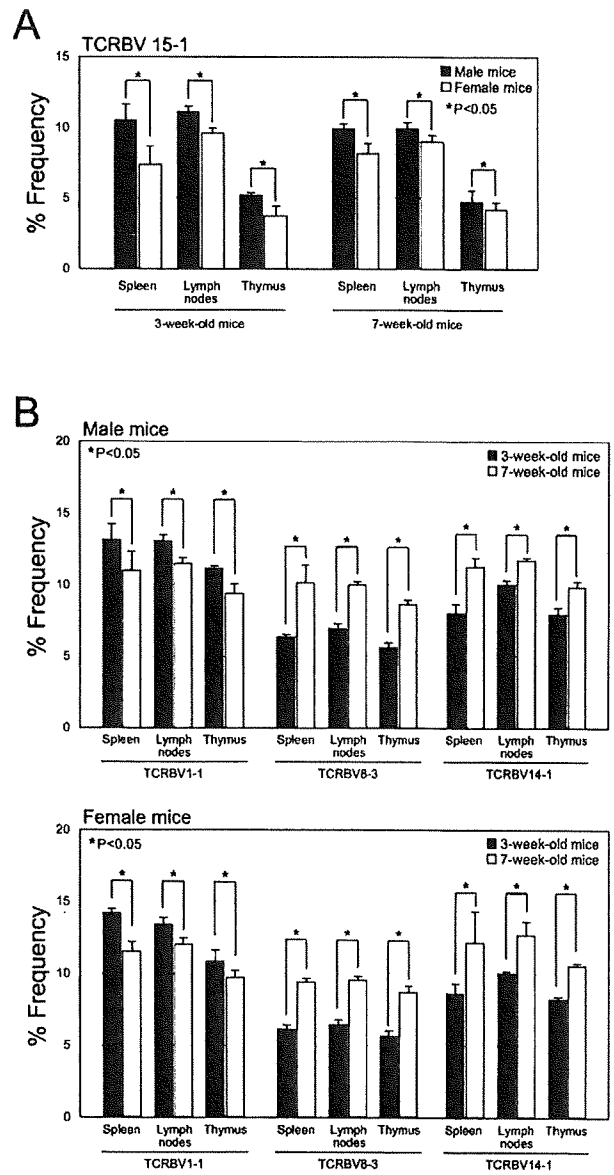

Fig. 2. Comparison of the TCR repertoires in mice of different sexes and ages. (A) Percentages of TCRBV15-1-bearing cells in the spleen, lymph nodes, and thymus of male (black bars) and female (white bars) mice at 3 weeks (left) and 7 weeks (right) of age. Vertical error bars indicate the standard deviation of five independent experiments. The TCR repertoires of these three organs were significantly higher in male mice than in female mice ($P<0.05$). (B) Percentages of TCRBV1-1-, 8-3-, and 14-1-bearing cells in the spleen, lymph nodes, and thymus of male (upper panel) and female (lower panel) mice at 3 weeks (black bars) and 7 weeks (white bars) of age. Vertical error bars indicate the standard deviation of five independent experiments. These three TCR repertoires differ significantly between the two ages ($P<0.05$).

Table 2. Changes in the percentages of CD4/CD8 T cell populations after administration of HC

	Number of CD3-positive cells ($\times 10^7$ cells)	Mean percentage of population ± SD			
		($CD4^+CD8^+$)	($CD4^+CD8^-$)	($CD4^-CD8^+$)	($CD4^-CD8^-$)
Thymus (PBS control)	32.5 ± 5.8	79.1 ± 0.7	10.6 ± 0.3	5.1 ± 0.5	5.2 ± 0.6
Thymus (HC)	2.9 ± 0.7[a)]	49.5 ± 9.6[b)]	26.1 ± 4.5[b)]	13.3 ± 2.2[b)]	11.0 ± 3.3[b)]
Spleen (PBS control)	21.1 ± 0.2	0.7 ± 0.1	67.4 ± 0.5	28.7 ± 0.6	3.3 ± 0.1
Spleen (HC)	14.4 ± 0.1[a)]	0.6 ± 0.1	67.6 ± 1.6	29.7 ± 1.2	2.1 ± 0.4

[a)]Number of CD3-positive cells differed significantly between PBS control and HC ($P<0.05$). [b)]CD4/CD8 population differed significantly between PBS control and HC in thymus ($P<0.05$).

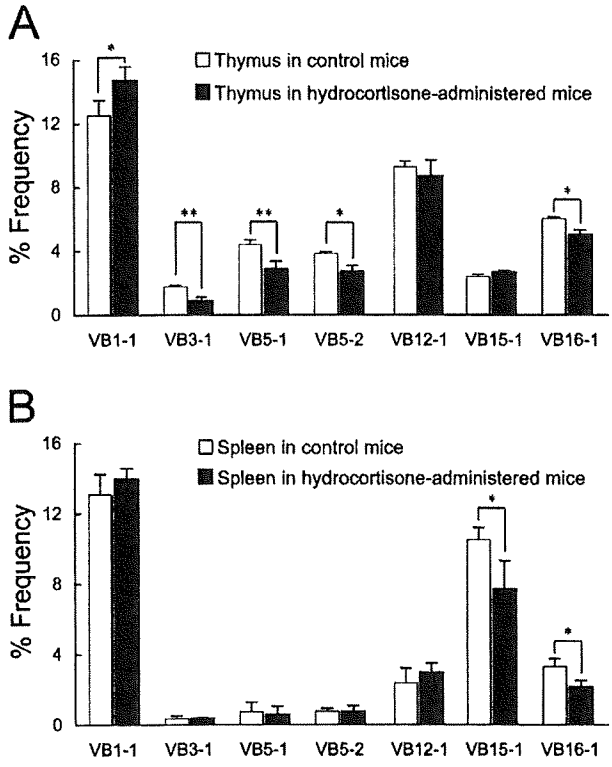

Fig. 3. Alterations in the TCR repertoires in the thymus and spleen after administration of HC. The percentages of cells bearing each TCRBV in control mice (white bars) and HC-administered mice (black bars) are shown. Panel (A) shows the TCRBV repertoires in the thymus. Panel (B) shows the TCRBV repertoires in the spleen. Vertical error bars indicate the standard deviation of five independent experiments. The families showing significant differences by Student's paired *t*-test are marked with asterisks (*$P<0.01$; **$P<0.005$).

$CD4^+CD8^+$ T cells, while $CD4^+CD8^-$ T cells and $CD4^-CD8^+$ T cells accounted for 11 and 5% of the cells, respectively. In contrast, in mice administered HC, $CD4^+CD8^+$ T cells, $CD4^+CD8^-$ T cells, and $CD4^-CD8^+$ T cells comprised 50, 26, and 13% of the thymic cells, respectively. The number of splenic T cells was decreased from 21.1×10^7 to 14.4×10^7 by HC. The CD4/CD8 T cell ratio, however, did not alter at all.

Alterations in TCR repertoire in the thymus and spleen induced by HC

The alterations observed in the TCR repertoire are shown in Fig. 3. The percentages of cells bearing TCRBV3-1, 5-1, 5-2, and 16-1 in the thymus significantly decreased in HC-treated mice compared with control mice. On the contrary, the percentage of cells bearing TCRBV1-1 in the thymus significantly increased in HC-treated mice. Cells bearing TCRBV15-1 and 16-1 in the spleen significantly decreased in HC-treated mice. The VB1-1 spleen T cells increased in number though not significantly.

Discussion

The thymus showed an unique TCRBV expression pattern among the organs examined in this study. Endogenous superantigens (SAGs) derived from mouse mammary tumor virus (MMTV) affect TCR repertoire by deleting T cells bearing particular VB chains either partially or completely in the thymus and peripheral lymphatic organs [2, 5, 21, 26]. BALB/c ($H\text{-}2^d$) mice express the Mtv-6, -8, and -9 genes from MMTV. The Mtv-6 SAG deletes $TCRBV3^+$ cells, while the Mtv-8 SAG deletes both $TCRBV11^+$ and 12^+ T cells [2, 21]. The Mtv-9 SAG induces clonal deletion of T cells bearing TCRBV5-1, 5-2, 11, and 12 [2, 21]. The expression patterns shown in the present study revealed that the percentages of virtually all these TCRBV subfamilies were low in peripheral lymphatic tissues, consistent with

the previous reports. TCRBV11$^+$ T cells, supposed to be deleted in BALB/c mice, however, were not deleted in the peripheral lymphatic tissues. We cannot think of an appropriate reason for this discrepancy. In addition, TCRBV16$^+$ T cells were incompletely deleted in the periphery, implying an alternative mechanism for the deletion of particular T cell clones other than SAGs.

The higher frequency of TCRBV15$^+$ T cells in lymph nodes and the spleen compared to the thymus suggest that another unknown peripheral positive selection or expansion mechanism is at work in the peripheral lymphatic organs.

Weak but detectable TCR signals were seen in the lung and intestinal tract. The lung, exposed to airborne foreign antigens through ventilation, possesses bronchus-associated lymphoid tissues (BALTs) [15]. Similarly, the intestinal tract, constantly exposed to food antigens and bacterial flora, has gut-associated lymphoid tissues (GALTs) [24]. Interestingly, in both the lung and the intestinal tract, although a TCR repertoire was observed that was generally consistent with that of the spleen and lymph nodes, TCRBV8-2$^+$ and 14$^+$ T cell numbers were decreased, whereas those of TCRBV19-1$^+$ and 20-1$^+$ T cells were increased. Since these organs have external milieu within and are exposed to external antigens, this condition might cause unique TCR repertoire changes (decrease of TCRBV8-2$^+$ and 14$^+$ T cells, and increase of TCRBV19-1$^+$ and 20-1$^+$ T cells). Unknown selection mechanisms may be functioning in the lung and in the intestinal tract. It was reported recently that an intestinal indigenous microbiota affect gene expression and cell activity of T cell, in a comparative study of SPF and germ free mice [9, 16]. The fact that the extent of expression of TCRBV19-1 and 20-1 in lymphocytes from the intestinal tract was greater than that from the lung may be due to the exposure to indigenous microbiota and/or food antigens. To address this issue, analyses with germ free animals would be gently helpful.

Sufficiently high signals for TCR analysis were not obtained for the liver, genital organs, the bone marrow and the brain. The relative CD3 mRNA expression levels were also low in these organs, suggesting that they contained only small T cell populations.

We further investigated whether the TCR repertoire would vary in normal mice according to sex and age. The frequency of TCRBV15-1-bearing cells was found to be higher in male than in female mice, irrespective of the age of the lymphatic tissues examined. The frequencies of TCRBV1-1-, 8-3-, and 14-1-bearing cells differed with age, with TCRBV8-3 and 14-1 higher showing in older mice.

The precise mechanisms behind these differences remain unknown. Various T cell selection systems, positive and/or negative, might be in operation systematically and/or locally, responding to genetic (sex) and/or environmental (tissue types and age) changes, leading to the alteration of the T cell repertoire. It is interesting, though, that indistinguishable age-dependent changes in the frequencies of TCRBV1-1-, 8-3-, and 14-1-bearing cells were found irrespective of the type of lymphatic tissue. In age-dependent changes, either an identical selection mechanism would operate in different lymphatic tissues including the thymus and lymph nodes, or similar genetic alterations would occur. Further extensive investigations of this are necessary.

HC-administered mice were found to have decreased numbers of CD4$^+$CD8$^+$ T cells in the thymus, suggesting that CD4$^+$CD8$^+$ T cells are HC-sensitive as previously reported [20]. The TCR repertoire in the thymus was also altered by HC treatment, as indicated by the decreased numbers of cells bearing TCRBV3-1, 5-1, 5-2, and 16-1. In peripheral T cells, MMTV sensitization causes clonal deletion of a proportion of the TCR families, including cells bearing TCRBV3-1, 5-1, 5-2, and 12-1 [2, 5, 21, 26]. In the present study, cells bearing TCRBV3-1, 5-1, and 5-2 in the thymus were found to be HC-sensitive. The numbers of these cells were greatly decreased, but they were not completely eliminated. In the spleen, however, these cell subsets were not significantly decreased by HC treatment. The reason for the increase in cells bearing TCRBV1-1 remains unknown, but a relative increase might have occurred due to a reduction in number of other TCRBV cells. On the other hand, one population, TCRBV12-1, partially deleted in the periphery after MMTV sensitization [2, 21], was found to be HC-resistant in this study. Cells bearing TCRBV16-1, which are unaffected by MMTV-sensitization, decreased in both the spleen and the thymus after HC administration. Therefore, TCRBV3-1, 5-1, and 5-2 are sensitive to both MMTV and HC, while TCRBV12-1

is sensitive only to MMTV and TCRBV16-1 is sensitive only to HC. We think that the sensitivity of TCR cells against MMTV or HC are totally disparate. Thus, HC, a potent endogenous stimulus, might create an entirely different environment within, leading to an alternative immune status of downregulating or overreacting to various antigens.

In the spleen, HC administration was found to decrease the number of T cells only slightly. Cells bearing TCRBV15-1 in the spleen, however, decreased significantly after HC administration. The counterpart subset in the thymus was not decreased by HC, suggesting TCRBV15-1 thymocytes are not identical to those in the spleen. The extent of deletion of some TCRBV families may be due to MMTV and/or other endogenous stimuli, like steroid hormones. In fact, completely deleted families were TCRBV3-1, 5-1, and 5-2, and partially deleted families were TCRBV12-1 and TCRBV16-1. It is possible that the difference of HC and/or MMTV sensibility in thymocytes influences the deletion level of peripheral T cells to suggesting the HC-resistant thymic lymphocyte is not equivalent to the medullary thymocyte, and that the HC-sensitive immature thymic lymphocyte is different among TCRBV families.

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Skew in T cell receptor usage with polyclonal expansion in lesions of oral lichen planus without hepatitis C virus infection

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Summary

Oral lichen planus (OLP) is a refractory disorder of the oral mucosa. Its predominant symptoms are pain and haphalgesia that impair the quality of life of patients. OLP develops via a T cell-mediated immune process. Here, we examined the characteristics of the infiltrating T cells in terms of the T cell receptor (TCR) repertoires, T cell clonality, T cell phenotypes and cytokine production profiles. TCR repertoire analyses and CDR3 size spectratyping were performed using peripheral blood mononuclear cells (PBMCs) and tissue specimens of OLP biopsies from 12 patients. The cytokine expression profiles and T cell phenotypes were measured by real-time quantitative polymerase chain reaction. We observed that there were skewed TCR repertoires in the tissue samples (TCRVA8-1, VA22-1, VB2-1, VB3-1 and VB5-1) and PBMCs (TCRVA8-1, VB2-1, VB3-1 and VB5-1) from OLP patients. Furthermore, the CDR3 distributions in the skewed TCR subfamilies exhibited polyclonal patterns. We observed increases in $CD4^+$ T lymphocytes, interleukin (IL)-5, tumour necrosis factor (TNF)-α and human leucocyte antigen D-related in the OLP tissue specimens. Taken together, the present results suggest that T cells bearing these TCRs are involved in the pathogenesis of OLP, and that IL-5 and TNF-α may participate in its inflammatory process.

Keywords: oral lichen planus, oral mucosa, superantigens, T lymphocytes, TCR repertoire

Introduction

Oral lichen planus (OLP) is a chronic inflammatory disease of the oral mucosa. Although the cause of OLP remains unclear, it is believed that certain patients with hepatitis C virus (HCV) infection and contact allergies to dental filling materials are susceptible to OLP [1]. Histopathologically, OLP is characterized by the presence of intraepithelial lymphocyte exocytosis, subepithelial band-like inflammatory infiltrates and hydropic degeneration of basal cells with apoptosis [2]. The infiltrating lymphocytes are composed predominantly of T cells with $CD4^+$ and $CD8^+$ phenotypes that express αβ T cell receptors (TCRs) [3]. $CD8^+$ T cells are found frequently adjacent to degenerated keratinocytes. Previously, several different TCR repertoires in lesions and peripheral blood mononuclear cells (PBMCs) of OLP patients have been reported, namely Vα2 and Vβ3 [4], Vβ13, Vβ14 and Vβ15 [5], Vβ2, Vβ16 and Vβ19 [6], and Vβ22 and Vβ23 [7]. Thus, the specific TCR repertoires in OLP lesions remain unclear.

T cells bearing TCRs recognize antigens in the form of peptide fragments in association with major histocompatibility complex (MHC) class I and class II molecules on antigen-presenting cells [8]. The fine specificity of T cells is determined by the TCRs displayed on the cell surface, which comprise heterodimers of an α-chain and a β-chain or a γ-chain and a δ-chain. The variable regions of these chains are responsible for antigen recognition and encoded by variable (V), joining (J) and diversity (D) gene segments. We have developed previously an adaptor ligation-mediated polymerase chain reaction (AL–PCR) method that allows us to define TCR repertoires based on the expression levels of transcripts, even when only small numbers of cells are available. This method enables us to amplify all the variable regions of the rearranged TCR genes through PCR cycles without skewing. Applying this method to a microplate hybridization assay (MHA) is simpler and more reproducible, and enables us to analyse TCR repertoires rapidly [9].

Random insertion of non-germinal element (N) nucleotides or deletion of nucleotides has been observed in the VN (D) NJ junction region designated CDR3, and is thought to be responsible for the antigenic peptide content [10,11]. Thus, any specific recognition of antigens by CDR3 can lead

Table 1. Pt data.

Patient	Age	Sex	Allergy	Past medical history
1	75	M	None	None
2	69	F	None	HT, arrhythmia, hyperlipidaemia
3	37	F	Food (soymilk)	Hyperlipidaemia, osteoporosis
4	64	M	None	None
5	65	M	None	HT
6	59	M	None	Tinea
7	78	F	None	Emphysema
8	38	M	None	None
9	65	M	None	None
10	70	F	None	HT, hyperlipidaemia
11	30	F	None	None
12	60	F	Pollinosis	HT, DM, hyperlipidaemia

HT, hypertension; DM, diabetes mellitus.

to clonal expansion of T cells. Furthermore, superantigens (SAs) produced by bacteria or viruses are known to bind to the outside of the MHC class II α-chain (TCRAV) and V-region of the TCR β-chain (TCRBV) to form a cross-link, such that a given SA can stimulate all T cells that bear the appropriate TCRBV in polyclonal settings [8]. Because CDR3 has different sequences and lengths, it is possible to analyse the diversity of TCRs by using a CDR3 size spectratyping method that provides a rapid scan of all TCR V-region transcripts grouped according to the utilized V-region gene and the chain length [12,13].

The aim of the present study was to elucidate the characteristics of the TCR repertoires in lesions and PBMCs of OLP patients without HCV infection using the AL–PCR and MHA methods and CDR3 spectratyping. We characterized further the T cell phenotypes and cytokine expression levels.

Materials and methods

Patients and samples

The OLP tissue specimens and peripheral blood were obtained from 12 patients with reticular type OLP at Tsurumi University Hospital and Yokohama Rosai Hospital (Table 1). The patient group comprised seven women and six men with an age range of 30–78 years (mean age: 59·2 years). None of the subjects had amalgam restorations or HCV infection. Informed consent was obtained from all patients. The study was approved by the local Research Ethical Committees at Tsurumi University, Yokohama Rosai Hospital and National Sagamihara Hospital. Lesional tissue specimens were taken from the buccal mucosa after biopsy for diagnostic purposes. Tissue specimens of normal oral mucosa (NOM) were obtained from six healthy volunteers. Control healthy peripheral blood samples were obtained from nine healthy volunteers, and age-matched to the patient group. The tissue specimens were obtained immediately after biopsy and soaked in RNAlater™ (Qiagen, Hilden, Germany). PBMCs were isolated from peripheral blood by H-SMF (Jimro Co. Ltd., Gunma, Japan) gradient centrifugation. The specimens were cryopreserved until RNA extraction.

The TCR repertoire analysis

Crude cellular RNAs from PBMCs and tissue specimens were extracted using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The AL–PCR and MHA methods were carried out as described previously [9]. Briefly, 1 μg of total RNA was reverse-transcribed to double-stranded cDNAs using a SuperScript II cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions, except for priming with a BSL-18B primer adaptor containing a Not I site. The P20EA/P10EA universal adaptors were ligated at the 5′ ends of the BSL-18B-primed cDNAs. Three rounds of Cα- and Cβ-specific PCR were performed using Cα and Cβ sequence-specific oligonucleotide probes (SSOPs), respectively, to prepare amplified and biotinylated TCR cDNA pools. Hybridization was carried out between biotinylated PCR products and AV or BV SSOPs immobilized on carboxylate-modified enzyme-linked immunosorbent assay plates (Sumitomo Bakelite, Tokyo, Japan). Positive hybridization was detected using p-nitrophenylphosphate (Nacalai Tesque, Osaka, Japan), and the visualized signals were estimated at 405 nm using a Multiskan JX Microplate Reader (Thermo Labsystems, Helsinki, Finland). Relative expansion of the TCRAV or TCRBV repertoire was calculated by the following formula: frequency (%) = X100 (corresponding SSOP signal)/(ΣTCRV SSOP signals).

CDR3 size analysis of TCRs

The above-described second PCR products were labelled by 20 cycles of PCR amplification with fluorescent dye-labelled C region-SSOPs [14]. After mixing with size markers (CEQ™ DNA Size Standard Kit-600; Beckman Coulter, Inc., Fullerton, CA, USA), the labelled PCR products were loaded onto a polyacrylamide sequencing gel (CEQTM Separation Gel-LPA I; Beckman Coulter, Inc.) to determine their sizes and fluorescence intensities using an automated capillary DNA sequencer (CEQ™ 8000; Beckman Coulter, Inc.). Data were analysed using the Genetic Analysis System Software (Beckman Coulter, Inc.).

Histology and immunohistochemistry

For histopathology, 6-μm sections of paraffin-embedded tissue specimens were stained with haematoxylin and eosin (H&E). Immunohistochemical studies were performed for the expression of CD3 (Dako, Kyoto, Japan), CD4 (Nichirei, Tokyo, Japan), CD8 (Nichirei), CD14 (Dako),

CD20 (Dako) and human leucocyte antigen D-related (HLA-DR) (Dako) using commercially available monoclonal antibodies following the ENVISION technique with 3,3-diaminobenzidinetetrahydrochloride as the indicator agent.

Real-time PCR

The mRNA expression levels of CD markers and cytokines were examined in PBMCs and tissue specimens by real-time quantitative PCR using a LightCycler® (Roche Diagnostics, Tokyo, Japan). Interleukin (IL)-2, IL-4, IL-5 and tumour necrosis factor (TNF)-α were examined in PBMCs and tissue specimens by real-time quantitative PCR using the LightCycler®. Total RNA was reverse-transcribed into cDNAs using a PrimeScript® RT Reagent Kit (Takara Bio, Shiga, Japan). One-tenth of each cDNA sample was amplified with a SYBR® Premix Ex Taq™ RT–PCR Kit (Takara Bio) according to the manufacturer's instructions. Primer pairs were obtained from Takara Bio. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression was used as an internal control. The results were expressed as the ratio of the amount of the respective cytokine-specific cDNA to the amount of GAPDH-specific cDNA.

Statistical analysis

Statistical analyses were carried out using StatView version 5·0 for Windows (SAS Institute, Cary, NC, USA). The non-parametric Mann–Whitney rank test was applied for all comparisons. Accordingly, the results of the comparisons are given as the mean or median. Values of $P < 0{\cdot}05$ were considered to indicate statistical significance.

Results

The TCR repertoire analysis

First, we examined the TCR repertoires in NOM tissue specimens and found that they varied markedly among individuals, with no significant differences from healthy PBMCs (Figs 1 and 2c). On the other hand, the TCR repertoires in OLP tissue specimens were quite similar, with significant increases in TCRAV8-1, AV22-1, BV2-1, BV3-1 and BV5-1 compared with healthy PBMCs ($P < 0{\cdot}05$) (Fig. 2a, b). Similarly, there were significant increases in TCRAV8-1, BV2-1, BV3-1 and BV5-1 in OLP PBMCs compared with healthy PBMCs ($P < 0{\cdot}05$) (Fig. 3). However, there were no significant differences in the TCR repertoires between OLP and NOM tissue specimens (Fig. 2c). The TCR repertoires in NOM tissue specimens varied markedly among individuals. The averages of the individual subfamilies seemed to increase, because the standard deviation values were very high. Therefore, the statistical differences were not significant even when we compared the frequencies in PBMCs and NOM tissue specimens.

T cell clonality in increased TCR repertoires

Using CDR3 size spectratyping, we further studied whether T cell clonality was present in the increased TCR repertoires, including TCRAV8-1, AV22-1, BV2-1, BV3-1 and BV5-1. The results revealed that all these TCR repertoires were composed of polyclonal expansion in OLP tissue specimens, whereas those in NOM tissue specimens showed monoclonal or oligoclonal patterns (Fig. 4). T cell clonality was confirmed by CDR3 size spectratyping. Among the TCR repertoires of OLP PBMCs, T cells bearing TCRAV8-1, BV2-1, BV3-1 and BV5-1 were subjected to CDR3 size spectratyping. The expansion of TCRV-bearing T cells showed polyclonal patterns in OLP PBMCs and healthy PBMCs (data not shown).

Histopathological features of NOM and OLP tissue specimens

The H&E staining of OLP tissue specimens revealed band-like infiltrates of lymphocytes in the superficial lamina propria and basement membrane destruction (Fig. 5). $CD3^+$, $CD4^+$ and $CD8^+$ T lymphocytes were detected among the infiltrating cells toward the basal membrane (Fig. 6a–c). NOM tissue specimens contained low numbers of $CD3^+$, $CD4^+$ and $CD8^+$ T lymphocytes (Fig. 6a–c). Expression of CD20 was detected in both OLP and NOM tissue specimens (Fig. 6d). HLA-DR expression was increased significantly in OLP tissue specimens compared with NOM tissue specimens (Fig. 6e). The $HLA\text{-}DR^+$ cells were considered to be antigen-presenting cells, as they were large compared with the lymphocytes.

Quantification of CD markers and cytokine expression levels

The mRNA expression levels of CD4, CD8, IL-2, IL-4, IL-5 and TNF-α in PBMCs and tissue specimens were measured by real-time quantitative PCR. GAPDH gene expression was used as an internal control. The mRNA expression levels represented by the ratio of the amount of each cytokine-specific cDNA to the amount of GAPDH-specific cDNA were compared between tissue samples or PBMCs. CD4 and CD8 were expressed in all samples (Fig. 7). In NOM and OLP tissue specimens, CD8 was expressed more highly than CD4 (Fig. 7a) ($P < 0{\cdot}005$). The CD4/CD8 ratio was higher in OLP tissue specimens than in NOM tissue specimens ($P < 0{\cdot}001$). There was no difference between the CD4/CD8 ratios in healthy PBMCs and OLP PBMCs (Fig. 7b). The mRNA expression levels of IL-2, IL-4, IL-5 and TNF-α were measured in healthy and OLP PBMCs and NOM and OLP tissue specimens to determine the cytokine environment in OLP patients (Fig. 8). The IL-5 and TNF-α expression levels were higher in NOM tissue specimens than in OLP tissue specimens ($P < 0{\cdot}001$). The IL-5

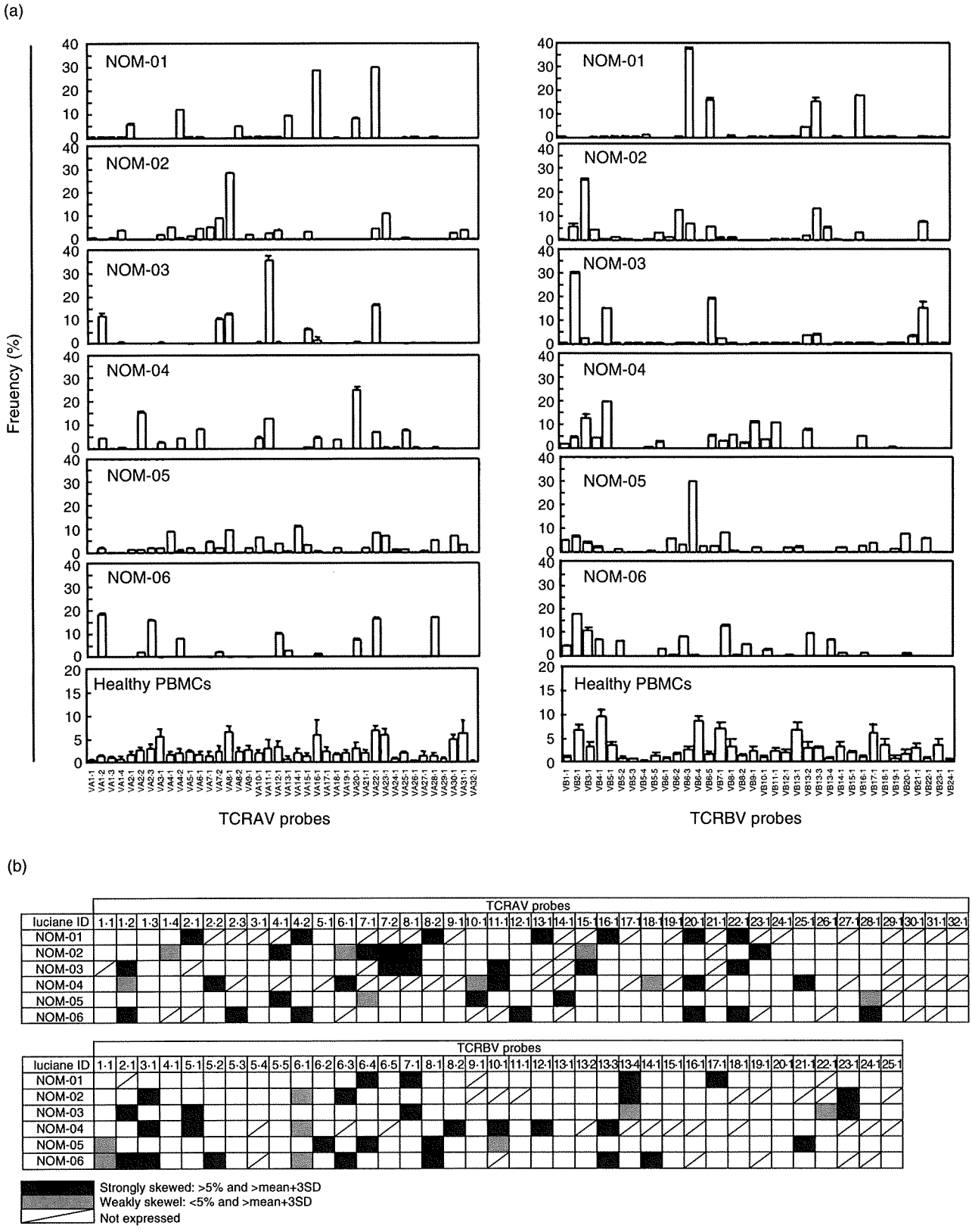

Fig. 1. Mean percentage frequencies of TCRVA and TCRVB in normal oral mucosa (NOM) tissue specimens from six healthy volunteers. The expressions of the different T cell receptor (TCR) repertoires in individual NOM tissue specimens are shown as mean ± standard deviation (a). TCRVA and TCRVB gene expression profiles in the NOM tissue specimens of healthy volunteers (b).

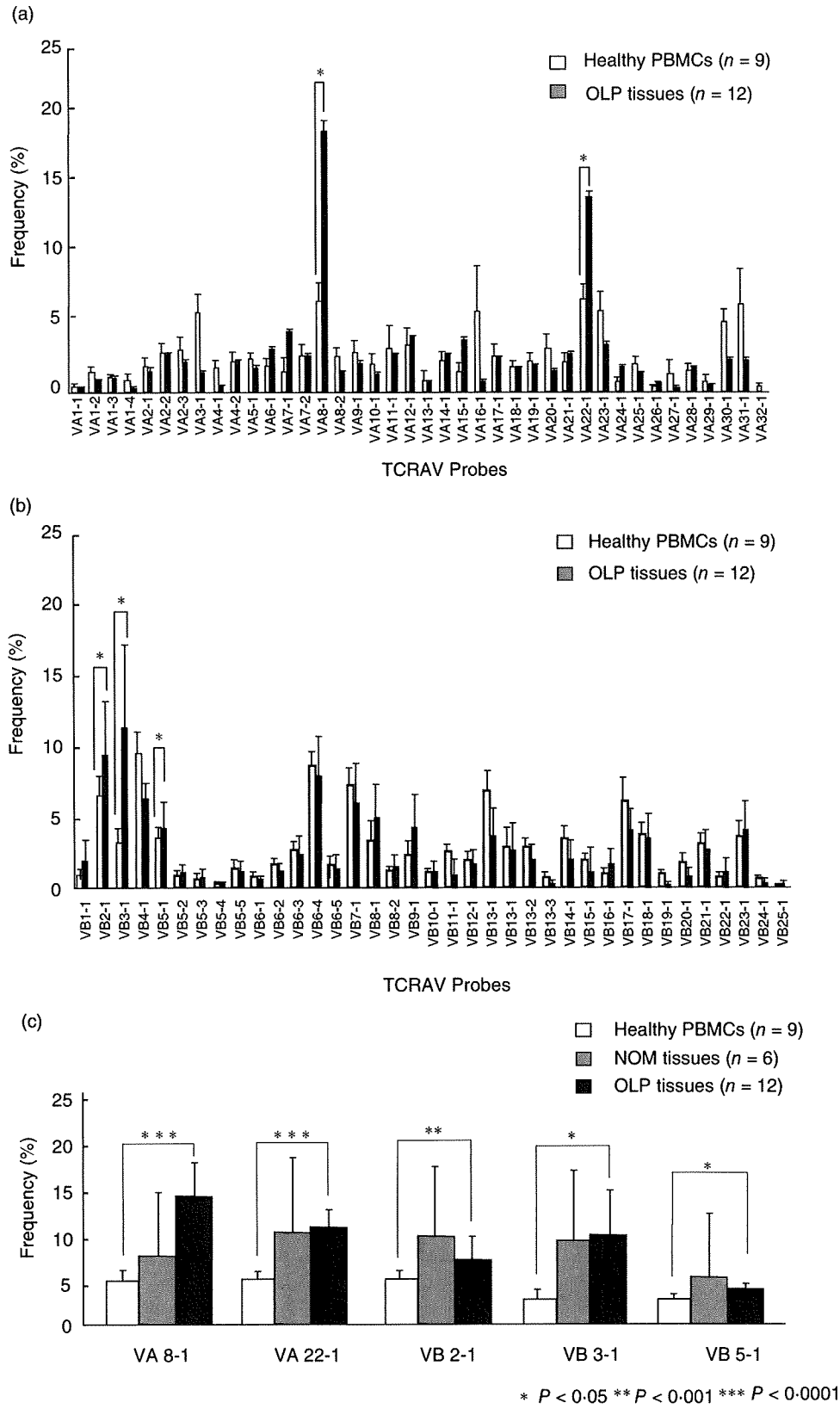

Fig. 2. The T cell receptor (TCR) repertoires in oral lichen planus (OLP) tissue specimens are skewed in comparison with those in healthy peripheral blood mononuclear cells (PBMCs). There are significant increases in TCRVA8-1 and VA22-1 (a) as well as VB2-1, VB3-1 and VB5-1 (b) in OLP tissue specimens compared with healthy PBMCs (*$P < 0{\cdot}05$). There are no significant increases in VA8-1, VA22-1, VB2-1, VB3-1 and VB5-1 in normal oral mucosa (NOM) tissue specimens compared with healthy PBMCs (c). All data are shown as mean ± standard deviation.

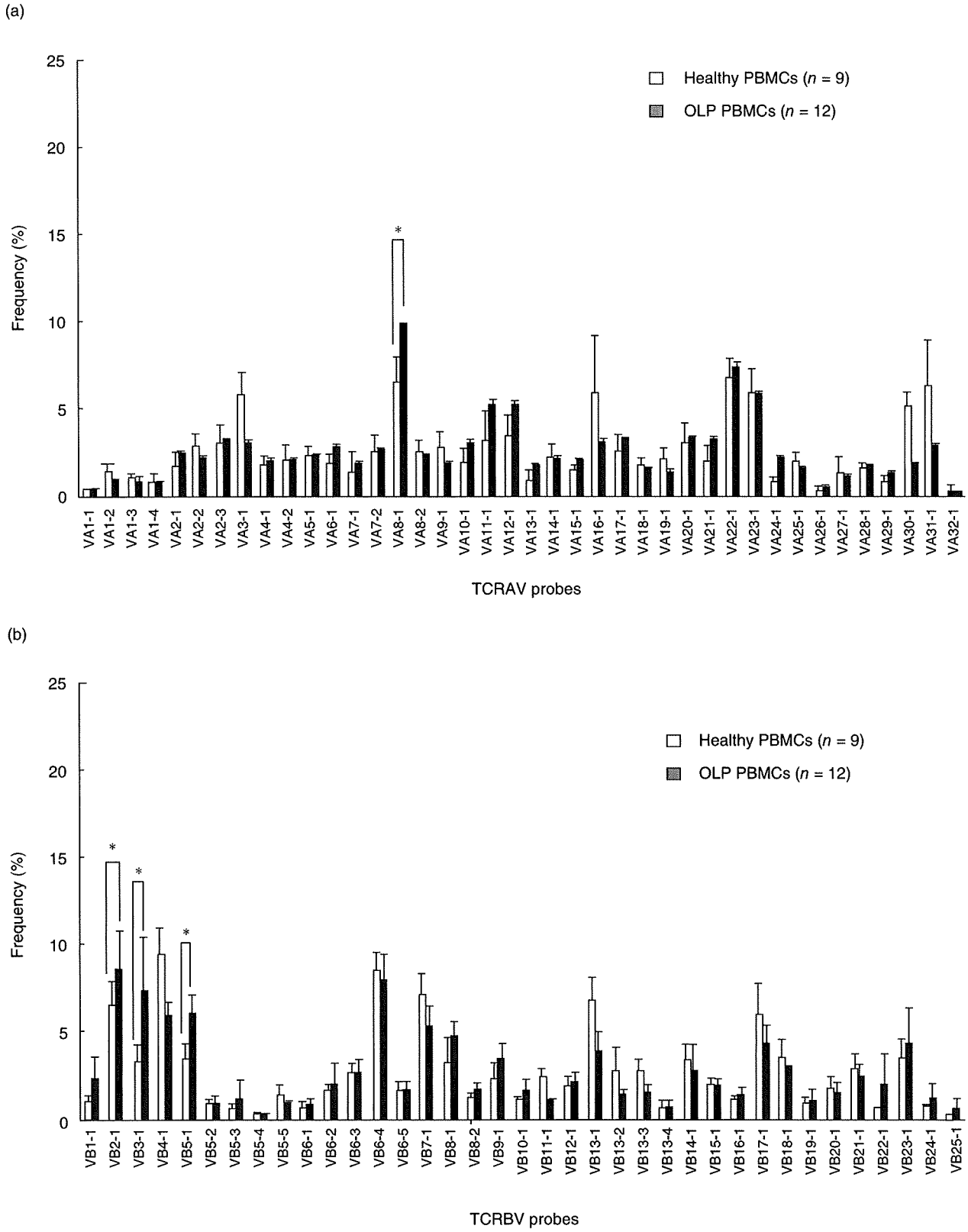

Fig. 3. The T cell receptor (TCR) repertoires in oral lichen planus (OLP) peripheral blood mononuclear cells (PBMCs) are skewed in comparison with those in healthy PBMCs. There are significant increases in T cells bearing TCRVA8 (a) as well as VB2, VB3 and VB5 (b) in OLP PBMCs compared with healthy PBMCs (*$P < 0{\cdot}05$).

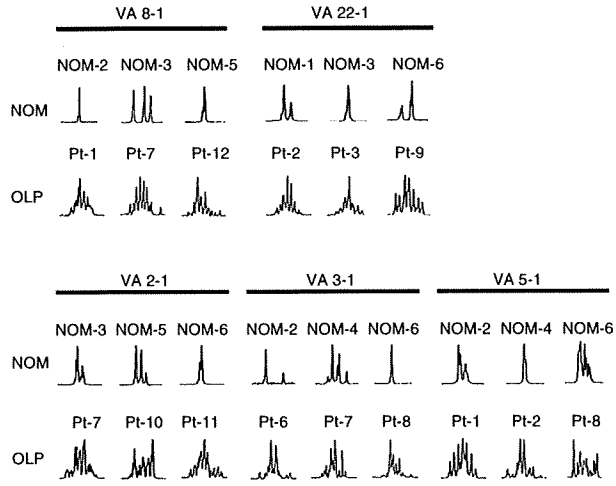

Fig. 4. Polyclonal expansion of T cell receptors (TCRs) within the oral mucosa of patients with oral lichen planus (OLP). Because the TCR repertoire analysis revealed skewed numbers of T cells bearing TCRVA8-1, VA22-1, VB2-1, VB3-1 and VB5-1, CDR3 size spectratyping was performed for all samples. In NOM tissue specimens, the CDR3 size distributions show monoclonal or oligoclonal patterns. In contrast, the CDR3 spectratyping patterns for OLP tissue specimens exhibit polyclonal or oligoclonal patterns.

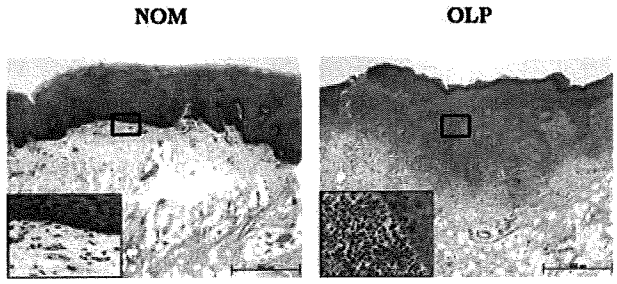

Fig. 5. Histopathological findings for normal oral mucosa (NOM) and oral lichen planus (OLP) tissue specimens. In NOM tissue specimens, lymphocytes are habitually present. In OLP tissue specimens, band-like infiltrates of lymphocytes in the superficial lamina propria and disruption of the basement membrane are detected. The insets show higher magnification images of the boxed regions. Magnifications: ×20 and ×200.

expression levels were higher in OLP PBMCs than in healthy PBMCs ($P < 0{\cdot}05$).

Discussion

Using the sensitive and reliable AL–PCR and MHA methods, we detected significant increases in TCR repertoires, namely TCRAV8-1, AV22-1, BV2-1, BV3-1 and BV5-1, in OLP tissue specimens compared with healthy PBMCs. In contrast, we detected variable TCRAV and TCRBV repertoires in NOM tissue specimens, with no particular specificity. Therefore, we consider that OLP lesions show a tendency towards

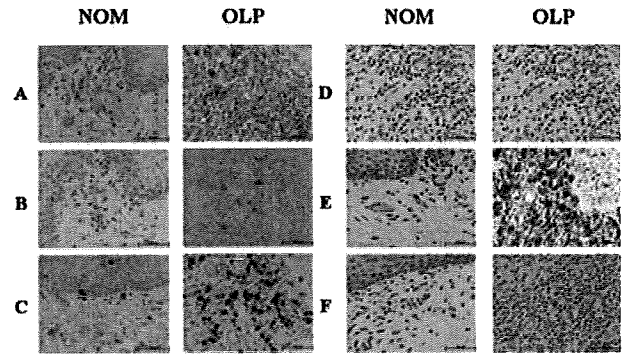

Fig. 6. Immunohistochemical staining for CD3 (a), CD4 (b), CD8 (c), CD20 (d) and human leucocyte antigen D-related (HLA-DR) (e) using commercially available monoclonal antibodies following the ENVISION technique with 3,3-diaminobenzidinetetrahydrochloride as the indicator agent. (f) Negative control. $CD3^+$, $CD4^+$ and $CD8^+$ T lymphocytes are detected among the infiltrating cells toward the basal membrane (a–c). Normal oral mucosa (NOM) tissue specimens contain low numbers of $CD3^+$, $CD4^+$ and $CD8^+$ T lymphocytes (a–c). Expression of CD20 is detected in both OLP and NOM tissue specimens (d). HLA-DR expression is increased significantly in OLP tissue specimens (e). Magnification: ×200.

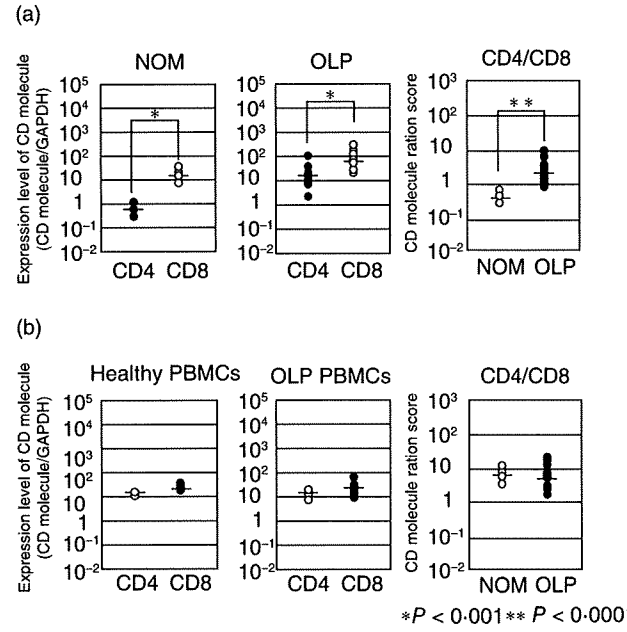

Fig. 7. Comparison of the expression levels of T cell phenotypes between normal oral mucosa (NOM) ($n = 6$) and oral lichen planus (OLP) ($n = 12$) tissue specimens (a) or healthy ($n = 9$) and OLP ($n = 12$) peripheral blood mononuclear cells (PBMCs) (b). The mRNA expression levels of $CD4^+$ and $CD8^+$ T cells were measured by real-time quantitative polymerase chain reaction (PCR). Each dot indicates a single sample. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression was used as an internal control. The expression levels of $CD8^+$ T cells are higher than $CD4^+$ T cells in NOM and OLP tissue specimens ($^*P < 0{\cdot}001$). The CD4/CD8 ratio is higher in OLP tissue specimens than in NOM tissue specimens ($^{**}P < 0{\cdot}0001$).

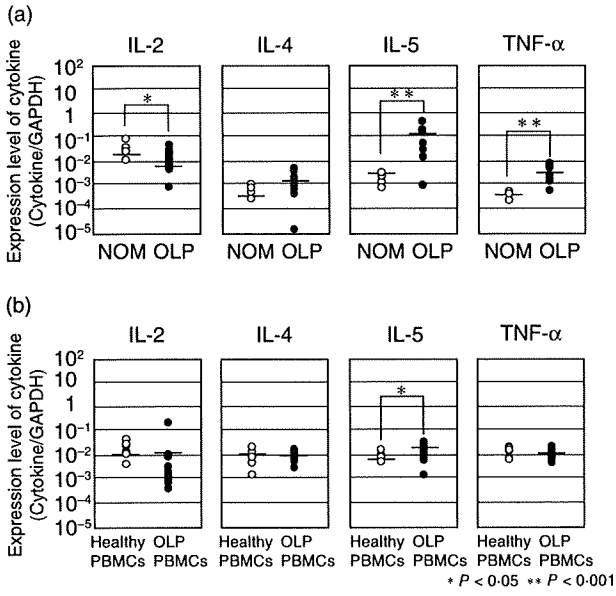

Fig. 8. Comparison of the expression levels of several cytokines between normal oral mucosa (NOM) ($n = 6$) and oral lichen planus (OLP) ($n = 12$) tissue specimens (a) or healthy ($n = 9$) and OLP ($n = 12$) peripheral blood mononuclear cells (PBMCs) (b). The mRNA expression levels of interleukin (IL)-2, IL-4, IL-5 and tumour necrosis factor (TNF)-α were measured by real-time quantitative polymerase chain reaction (PCR). Each dot indicates a single sample. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression was used as an internal control. The mRNA levels expressed by the ratio of the amount of the each cytokine-specific cDNA to the amount of GAPDH-specific cDNA were compared between NOM and OLP tissue specimens or healthy and OLP PBMCs. (a) The expression levels of IL-5 and TNF-α are higher in OLP tissue specimens than in NOM tissue specimens. The IL-2 expression levels are lower in OLP tissue specimens than in NOM tissue specimens. (b) The IL-5 expression levels are higher in OLP PBMCs than in healthy PBMCs.

increased infiltration of T cells with these TCR repertoires. The observation that increased usage of TCRAV8-1, BV2-1, BV3-1 and BV5-1 was also detected in OLP PBMCs suggests that T cells with these TCR repertoires expand into the blood and infiltrate the lesional mucous membrane, where they may participate in the inflammatory process.

Previous studies have reported that usage of TCRAV2, BV2, BV3, BV6, BV13, BV14, BV15 and BV19 is increased in OLP lesions and that BV22 and BV23 are increased in *in vivo*-expanded cells [4–6]. Our observation that TCRBV3-1 was increased in OLP lesions is compatible with a previous observation, that T cells bearing TCRBV3 are increased in OLP lesions as evaluated by immunohistochemistry [4]. The increased usage of TCRBV2-1 in OLP lesions detected in the present study is compatible with a previous finding of increased usage of TCRBV2 detected by semiquantitative PCR [6]. In the present study, we did not find increased usage of TCRAV2, BV6, BV13, BV14, BV15 or BV19. The discrepancy between our results and previous findings may be attributed to differences in the detection methods or the series of examined patients. Because the AL–PCR and MHA methods are highly reliable for quantitative evaluation of TCR usage [9], we believe that the present findings are more reliable than the previously reported findings. We examined reticular-type OLP patients without HCV infection, whereas previous reports included erosive and atrophic types. It is unclear whether the patients in the previous reports were associated with HCV infection. Differences in the characteristics of the examined patients in the present and previous studies may represent another possible reason for the differing results.

In the present study, we have demonstrated a striking difference in the clonality of TCR repertoires between NOM and OLP tissue specimens by CDR3 size spectratyping. The TCR repertoires of TCRAV8-1, AV22-1, BV2-1, BV3-1 and BV5-1 appeared to be polyclonal in OLP tissue specimens, but oligoclonal in NOM tissue specimens. These findings suggest that the band-like infiltrates in OLP lesional mucosa are composed of polyclonally expanded T cells. It has been suggested that SAs are involved in the pathogenesis of OLP [6]. In fact, TCRBV2-1, BV3-1 and BV5-1 are known to be activated by various SAs, including TSST-1, SEB, SEC3, SED and SEE [5]. The pattern of TCR usage in T cells expanded by each SA is usually restricted. For instance, TCRBV2 and BV4 are expanded by TSST-1, while TCRBV1, BV5, BV6, BV7, BV9 and BV18 are expanded by SEA [15,16]. Andersen *et al.* [17] reported preferential expression of certain Vα regions among SA-reactive T cells and suggested that the TCR-α chain may modulate the level of activation through an interaction with MHC. In addition, Petersson *et al.* [18,19] reported that *Staphylococcal enterotoxin H* induces TCR Vα27-specific expansion of T cells. There are no previous reports of SAs that induce specific expansion of T cells bearing TCRAV8-1, AV22-1, BV2-1, BV3-1 or BV5-1, suggesting that the pattern of TCR usage expansion observed in the present study may not be related to a particular SA or that a currently unknown SA is involved in OLP pathogenesis. It has been suggested that T cell clones are deleted in peripheral blood by some SAs [20,21]. In the present study, there were significant decreases in T cells bearing VA3-1, VA16-1, VA31-1, VA32-1 and VB4-1 in the OLP PBMCs compared with healthy PBMCs. These observations support further the hypothesis that SAs may be contribute to OLP pathogenesis, and that T cells with limited TCR repertoires are involved in its inflammatory process.

Previous studies have indicated that autoimmunity may be involved in the pathogenesis of OLP. The lichenoid tissue reactions observed in OLP lesions resemble the histological patterns of graft-*versus*-host disease [1]. Lichen planus-like lesions in oral mucosa have been described in patients after autologous bone marrow transplantation [22,23]. Peripheral T lymphocytes from OLP patients have been shown to induce cell lysis of oral epithelial cells [24]. In addition, injection of Ia-reactive T cell clones into syngeneic mice

causes lichenoid epidermal changes in an experimental model [25]. It is thus possible that autoimmune mechanisms may be involved in the pathogenesis of OLP. Interestingly, a recent study revealed that TCRAV22 is expressed by T cell clones specific for desmoglein-3, a pemphigoid antigen [26]. Desmoglein-3 is a known surface molecule of basal cells in the oral epithelium, implying that T cells bearing TCRAV22 may cause inflammation of the mucous membrane by responding to this molecule on the basal keratinocytes. The issue of whether autoreactive T cells participate in the pathogenesis in OLP remains to be clarified in future studies. In the present study, expansion of TCRVA22-1-bearing T cells was observed distinctively in OLP lesions. This observation raises another possibility that T cells activated by SAs may recognize self-antigens, due probably to cross-reactivity.

While the majority of intraepithelial lymphocytes in OLP are $CD8^+$ cytotoxic T cells, most lymphocytes in the lamina propria are $CD4^+$ helper T cells [27,28]. In the present study, $CD8^+$ cytotoxic T cells were found significantly in the lamina propria and the mRNA expression levels in $CD4^+$ and $CD8^+$ T lymphocytes were increased in OLP. These findings suggest that $CD4^+$ T cells are increased in OLP lesions, as the CD4/CD8 ratio was higher in OLP tissue specimens than in NOM tissue specimens. Therefore, investigation of the TCR repertoires of $CD8^+$ T cells infiltrating OLP lesions may lead to a better understanding of OLP pathogenesis.

The finding that TNF-α expression was increased in OLP tissue specimens compared with NOM tissue specimens is compatible with a previous report [29]. Surprisingly, we found increased levels of IL-5 expression, but not IL-4 expression, in OLP tissue specimens and PBMCs. It has been suggested that mast cells and regulated upon activation normal T cell expressed and secreted (RANTES) may play roles in OLP pathogenesis [30]. IL-5 is known to be important for mast cell survival, and may therefore be involved in the mast cell-dependent inflammatory process.

In conclusion, we have observed skewed TCR repertoires in tissue specimens and PBMCs from OLP patients. Furthermore, the CDR3 distributions of the skewed TCR subfamilies exhibited polyclonal or oligoclonal patterns. Thus, polyclonal T cell expansion in the blood and mucous lesions may be induced in individuals with OLP. T cells responding to SAs, certain self-antigens or external antigens may be involved in the pathogenesis of OLP. Further analyses are required to understand fully the exact aetiology of OLP.

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Bacterial superantigens and T cell receptor β-chain-bearing T cells in the immunopathogenesis of ulcerative colitis

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Summary

Ulcerative colitis (UC) is a chronic relapsing–remitting inflammatory bowel disease (IBD) that affects the colon and the rectum producing debilitating symptoms, which impair ability to function and quality of life. The aetiology of IBD is incompletely understood, but within the lymphocyte population, specific T cell subsets are known to be major factors in the development of intestinal immune pathology while different subsets are essential regulators, controlling IBD. Hence, IBD is thought to reflect dysregulated T cell behaviour. This study was to investigate if the normal molecular configuration of the T cell receptor (TCR) repertoire is compromised in patients with UC. The percentage of T cell-bearing β-chain 4 (TCRBV4) was high in patients with UC, and T cells showed polyclonal expansion in the presence of bacterial superantigens (SA) such as streptococcal mitogenic exotoxin Z-2 (SMEZ-2), indicating that bacterial SA promote specific TCRBV family expansion. Further, in patients with UC, the duration of UC was significantly longer in patients with skewed TCRBV4 compared with patients without TCRBV4 skewing, suggesting that long-term exposure to bacterial SA such as SMEZ-2 might promote systemic immune disorders like the remission-relapsing cycles seen in patients with UC. In conclusion, our observations in this study support the perception that the systemic activation of T cells by enteric bacterial SA might lead to a dysregulated, but exuberant immune activity causing the remission and flare-up cycle of mucosal inflammation in patients with UC. Future studies should strengthen our findings and increase understanding on the aetiology of IBD.

Keywords: bacterial superantigens, inflammatory bowel disease, T lymphocytes, TCR repertoire, ulcerative colitis

Introduction

Ulcerative colitis (UC) is a chronic relapsing–remitting inflammatory bowel disease (IBD) that affects the colon and the rectum and produces debilitating symptoms, which impair both ability to function and quality of life [1]. At present, factors which exacerbate and perpetuate UC are not well understood. Multiple factors, including environmental influences [2–4], enteric flora [1,5–7] and genetic susceptibility [8], are thought to contribute to the dysregulated immune function [9,10] seen in patients with IBD. However, active UC is associated frequently with infiltration of a vast number of leucocytes, mainly activated T cells, monocytes/macrophages and polymorphonuclear cells, into the intestinal mucosa [1,11–15]. In line with these observations, several studies have suggested that T cells are major players in the immunopathogenesis of UC [9,12–18]. Accordingly, certain immunosuppressants such as cyclosporin A, a T cell-specific immunosuppressive agent, has been used to induce remission of active UC [19]. Given that certain T cell subsets such as the $CD4^+CD25^+$ phenotype (known popularly as regulatory T cells) are involved intimately in the control of intestinal immune pathology [20,21], it is logical to look for factors which are associated with dysregulated T cell features in the peripheral blood and within the intestinal mucosa of patients with UC.

T cells are known to recognize the antigen presented by antigen-presenting cells through the T cell receptor (TCR) in the context of major histocompatibility complex (MHC) class I and class II molecules [22]. The fine specificity of T cells is determined by the TCR displayed on the cell surface, a heterodimer composed of an α-chain and a β-chain or a γ-chain and a δ-chain. The variable regions of these chains are responsible for antigen recognition and are encoded with variable (V), joining (J) and diversity (D) (for the β-chain) gene segments. Random insertion of non-germinal element (N) nucleotides or deletion of nucleotides has been observed in the VN(D)NJ junction region called CDR3, and is thought to be responsible for an antigenic peptide content [23,24]. Thus, any specific recognition of antigens by CDR3 can lead to the clonal expansion of T cells. Further, it has been known that superantigens (SA) such as staphylococcal enterotoxins and streptococcal pyrogenic exotoxins produced by bacteria bind to the outside of the MHC class II α-chain and V region of TCR β-chain (TCRBV) to form a cross-linking; a given SA can stimulate all T cells that bear the appropriate TCRBV in polyclonal settings [22]. As CDR3 has different sequences and lengths, it is possible to analyse the diversity of the TCRs by using the CDR3 size spectratyping method that provides a rapid scan of all TCRBV transcripts, grouped according to the utilized Vβ gene and the lengths of the chains [25,26]. Using this technique with TCR repertoire analysis, it is possible to investigate the diversity of TCRs [27–29]. By using TCR repertoire analysis and CDR3 spectratyping, this study aimed to gain further understanding on T cell profiles associated with UC, to investigate if the normal molecular configuration of the TCR repertoire of T cells is compromised in patients with UC.

Materials and methods

Test samples

Peripheral blood samples were obtained from 22 patients with active UC and 20 healthy controls after obtaining informed consent at the Sakura Medical Center of Toho University (Japan). The study protocol was reviewed and approved by the local Committee on Ethics of experiments involving humans. Peripheral blood mononuclear cells (PBMC) were separated from heparinized blood using lymphocyte separation medium (H-SMF; Jimro, Gunma, Japan) gradient centrifugation. PBMC were washed with RPMI-1640 (Invitrogen, Carlsbad, CA, USA) and used in several experiments. The plasma samples were kept frozen until assay. In an additional investigation, colonic biopsies from five patients were processed. In each case, two or three small macroscopically inflamed mucosal biopsy specimens were obtained at colonoscopy. The biopsy samples were soaked in RNA*later*™ (Qiagen, Hilden, Germany) and kept frozen until use. Peripheral blood specimens were also obtained from these five patients.

Stimulation of PBMC with recombinant streptococcal mitogenic exotoxin Z-2 (rSMEZ-2) and recombinant toxic shock syndrome toxin 1 (rTSST-1)

PBMC were incubated with 1 ng/ml of rTSST-1 (Toxin Technology, Sarasota, FL, USA) or with 20 pg/ml of rSEMZ-2 (a kind gift from Dr John D. Fraser, University of Auckland, New Zealand) at 1×10^6 cells/ml for 3 days. After 3 days, 20 ng/ml of interleukin (IL)-2 (Shionogi, Osaka, Japan) was added to each well and the cells were incubated for a further 24 h (for rSMEZ-2 stimulation only). The cells were harvested and used for analysis of the TCR repertoire.

Analysis of TCR repertoire

Crude cellular RNA from PBMC, stimulated PBMC or biopsy samples was extracted by using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Adaptor-ligation polymerase chain reaction (PCR) and microplate hybridization assay were performed as previously described [30]. Briefly, 1 μg of total RNA was converted to a double-stranded cDNA with the SuperScript II cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions, except for priming with BSL-18B primer adaptor containing the *Not I* site. The P20EA/P10EA universal adaptors were ligated at the 5′ end of BSL-18B primer cDNA. Three rounds of Cα- and Cβ-specific PCR were performed by using Cα and Cβ sequence-specific oligonucleotide probes (SSOP) to prepare amplified and biotinylated TCR cDNA pools. Hybridization was between biotinylated PCR products and Vα or Vβ SSOP, which were immobilized on a carboxylate-modified enzyme-linked immunosorbent assay (ELISA) plate (Sumitomo Bakelite, Tokyo, Japan). The hybridization was visualized by *p*-nitrophenylphosphate (Nacalai Tesque, Osaka, Japan). The visualized signals were estimated at 405 nm using Multiskan JX (Thermo Labsystems, Helsinki, Finland). The relative expansion of the TCRAV or TCRBV region repertoire was calculated by the following formula: frequency (%) = 100 (the corresponding SSOP signal)/(ΣTCRV SSOP signals).

CDR3 size analysis of TCRBV

By knowing the size of the CDR3 region in TCRBV, it is possible to estimate the polyclonal expansion of T cells [31]. We used this technique to determine the polyclonality of T cells from patients with active UC who had skewed TCRBV4 and PBMC from healthy controls before and after *in vitro* stimulation with SA. The second PCR products described above were labelled by the 20-cycle PCR amplification with fluorescent dye-labelled Cβ–SSOP [31]. After the labelled PCR products were mixed with size marker (CEQ™ DNA Size Standard Kit-600; Beckman Coulter, Fullerton, CA, USA), they were loaded onto a polyacrylamide sequencing

