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Analysis of peripheral blood mononuclear cell stimulated with pyruvate dehydrogenase complex, T-cell receptors from patients with primary biliary cirrhosis

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Editorial on page 779

Abstract: Progressive destruction of the intrahepatic bile ducts in patients with primary biliary cirrhosis (PBC) is thought to be mediated by cytotoxic T cells which recognize certain epitopes, such as the pyruvate dehydrogenase complex (PDC). To clarify the T-cell repertoire in PBC, we analyzed T-cell receptor (TCR) Vβ-chain messages expressed in peripheral blood mononuclear cells (PBMCs) stimulated with PDC and in liver biopsy specimens. PBMCs from 12 PBC patients and 6 healthy controls were examined. The TCR Vβ repertoires of unstimulated PBMCs and PBMCs stimulated with PDC purified from bovine heart were analyzed, using the reverse transcriptase-polymerase chain reaction (RT-PCR) and single-strand conformation polymorphism (SSCP). Liver biopsy specimens from 5 PBC patients were also analyzed. In the PBC patients, several different T-cell clones, some of which showed the same mobility, were evident in both the PDC-stimulated and unstimulated PBMCs, as demonstrated by SSCP analysis. In addition, TCR clonality of infiltrating lymphocytes in the liver was also observed in PBC patients, showing common clonal T-cell accumulation with that seen in PBMCs stimulated with PDC. These data indicate that common clonal T-cell accumulation specific for PDC may be present in both peripheral PBMCs and the liver of patients with PBC.

Key words: T-cell receptor, pyruvate dehydrogenase complex, primary biliary cirrhosis, single strand conformation polymorphism

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Introduction

Primary biliary cirrhosis (PBC) is an idiopathic liver disease characterized by progressive destruction of intrahepatic bile ducts and possibly caused by autoimmune reactions. Although the abundance of immunologic abnormalities in patients with PBC suggests that the disease is caused by some abnormality of immune regulation, its pathogenesis remans unknown.[1] The destruction of the biliary tract in patients with PBC has recently been considered to be mediated by cytotoxic T cells which recognize certain epitopes, such as the pyruvate dehydrogenase complex (PDC), of the inner membrane of mitochondria.[2] PDC is a multienzyme complex which consists of five components: E2, Protein X, E3, E1-α, and E1-β,[3] and T-cell responses to PDC-E2/Pro-X are reported to be strongly associated with PBC.[4] To date, T-cell clones specific for PDC-E2 have been reported in peripheral blood and in liver.[5,6] However, whether these T-cell clones are truly involved in the pathogenesis of PBC has yet to be clarified.

During an antigen-specific immune response, T cells recognize the antigen via the T-cell receptor (TCR) and those T cells that bear the same sequence in the complementarity-determining region 3 (CDR 3) are selected and expanded upon stimulation by a specific antigen. Thus, an antigen-specific immune response can be interpreted by analysis of the CDR 3 of the TCR involved. TCRs consist of α and β chains and each T-cell clone exhibits a distinct set of α and β chains.[7,8] The β chain is composed of the V, D, J, and C regions and has three hypervariable regions that are responsible for binding with antigen peptides.[8] TCR Vβ clonality in other autoimmune diseases has been investigated in order to determine the response of T cells to specific antigens, by amino acid sequencing in the CDR3 regions,[9,10] and by single-strand conformation polymorphism (SSCP) analysis of TCR Vβ.[11,12]

Studies of a small number of T-cell clones generated from the liver tissue of PBC patients have indicated oligoclonality by virtue of identical TCR-chain gene rearrangements.[6,13–15] Recently, Ohmoto et al.[16] used SSCP analysis to demonstrate that multiple T-cell clonotypes accumulate in the liver of patients with PBC, with some identical T-cell clones accumulating in the peripheral blood. However, an analysis of TCRs stimulated with PDC has yet to be performed. The objectives of the present study were to clarify the clonality of T cells in peripheral blood mononuclear cells (PBMCs) obtained from PBC patients and to determine whether there are common T-cell clones in the PDC-stimulated PBMCs and the liver tissue of patients with PBC. To this end, we used the highly sensitive SSCP method to examine the TCR Vβ repertoire from Vβ 1 to Vβ 20.

Patients and methods

Patients

The subjects were 12 patients with PBC (Scheuer stage I, $n = 4$; stage II, $n = 4$; stage III, $n = 2$; stage IV, $n = 2$) treated at II Department of Internal Medicine, Fukushima Medical College Hospital, Japan. A diagnosis of PBC was made if one of the following was present:[17] (1) pathohistological features of chronic nonsuppurative destructive cholangitis, (2) a positive result on antimitochondrial antibody (AMA) testing, with pathohistological changes compatible with PBC, (3) a positive AMA result and clinical manifestation of the symptoms and course of PBC despite a lack of pathohistological features.

Preparation of mononuclear cells from peripheral blood

Mononuclear cells from heparinized venous peripheral blood were obtained from the 12 PBC patients and six healthy controls by standard density gradient centrifugation, using Lymphoprep (Nyegaard, Oslo, Norway).[18] The cells in the interphase layer were washed three times in buffered Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco BRL, Life Technologies, Rockville, MD, USA) supplemented with 2 mM L-glutamine, 100 mg/ml streptomycin, 200 U/ml penicillin, and 2 mg/ml amphotericin B. PBMC viability was determined by the trypan blue dye exclusion method and cell populations that were more than 97% viable were used for the experiments. The PBMCs were resuspended in buffered RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (Gibco BRL) to a final concentration of 3×10^6 cells/ml.

Purification of pyruvate dehydrogenase complex (PDC)

PDC was purified from bovine heart, using the method of Stanley and Perham.[19] Briefly, bovine heart was cut into cubes and suspended in 50 mM Mops (4-morpholine-propanesulfonic acid), pH 7.0, containing 2.7 mM ethylene diamine tetraacetic acid (EDTA), 0.1 mM dithiothreitol, and 3% Triton-X 100, and blended in a Waring blender. The homogenate was then centrifuged by differential centrifugation-precipitation with 35% polyethylene glycol at 25 000 *g* for 10 min. The precipitate was resuspended in 50 mM Mops buffer and centrifuged at 40 000 *g* for 60 min. The supernatant was collected, the pH adjusted to 5.4 with 10% acetic acid, and centrifugation was done at 25 000 *g* for 10 min. The resultant supernatant contained PDC and α-ketoglutarate dehydrogenase complex. PDC was purified by gel filtration at 4°C, using a Sepharose CL-2B column (Pharmacia, Uppsala, Sweden). The purity of the PDC preparation, as evident in Fig. 1, was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described.[20] This preparation was then used to stimulate PBMCs.

Optimal concentration of pyruvate dehydrogenase complex required to stimulate mononuclear cells in peripheral blood

To determine the optimal PDC concentration for adequate stimulation of PBMCs, freshly isolated PBMCs (3×10^6 cells/ml $\times$ 200 µl) were cultured in flat-bottomed sterile 96-well microtiter plates (Nunclon; Gibco BRL) alone or with purified PDC at 3, 3×10^{-1}, 3×10^{-2}, 3×10^{-3}, or 3×10^{-4} µg/ml, at 37°C for 3 days in a 100% humidified atmosphere of 5% CO_2. A 1-µCi aliquot of [^{3}H]thymidine was then added to each well and the plates were incubated for 18 h. Radioactivity in harvested cells was measured with a scintillation counter (ALOKA, Tokyo, Japan). The stimulation index (SI) was calculated: this was the ratio of [^{3}H]thymidine uptake by PBMCs stimulated with PDC to uptake by unstimulated PBMCs.

Stimulation of PBMCs with PDC

Freshly isolated PBMCs (3×10^6 cells/ml $\times$ 2 ml) were cultured in a 24-well flat-bottomed microtiter plate (Nunclon; Gibco BRL) with purified PDC at 3×10^{-1} µg/ml in complete medium (RPMI supplemented with 10% fetal calf serum) for 3 days at 37°C in a 100% humidified atmosphere containing 5% CO_2. A portion of each of these samples was set aside for RNA extraction. A 200-µl aliquot of each sample was then trans-

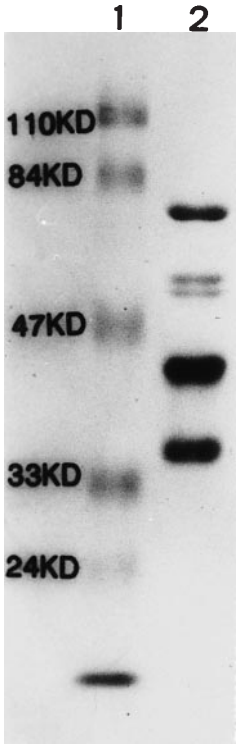

Fig. 1. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis of pyruvate dehydrogenase complex (PDC) preparation. *Lane 1*, Marker proteins; *lane 2*, purified sample; five bands are evident: 74 kD (E2), 55 kD (E3), 50 kD (protein X), 42 kD (E1-α) and 37 kD (E1-β)

ferred to a 96-well plate, and 1 μCi [^{3}H]thymidine was added to each well. After incubation for 18 h, the cells were harvested and washed with buffered RPMI 1640 medium, and radioactivity was measured with a scintillation counter (ALOKA). The SI was calculated as described above. An SI higher than the mean + 2SD of the SI for the healthy controls was considered to be a positive response to antigen.

Liver biopsy specimens

Liver specimens were obtained from 5 (stage I, $n = 1$; stage II, $n = 1$; stage III, $n = 2$; stage IV, $n = 1$) of the 12 PBC patients by percutaneous needle biopsy.

Isolation of RNA from PBMCs and liver tissue and reverse transcription polymerase chain reaction

Total RNA was isolated from cells and liver tissue by Chomczynski's method.[21] PBMCs cultured with or without PDC, and the liver biopsy specimens were suspended separately in Isogen-LS solution (Nippon Gene, Tokyo, Japan), mixed with chloroform and centrifuged. RNA, precipitated from the aqueous phase by the addition of isopropanol, was then washed and dissolved in water or stabilized with formamide. Complementary DNA (cDNA) was synthesized in a reaction containing the extracted RNA, reverse transcriptase (Takara Biotechnology, Tokyo, Japan), 10 mM Deoxyribonucleoside triphosphate solution (dNTP) (Gibco) and a random primer (Gibco). The cDNAs obtained were amplified by the polymerase chain reaction (PCR), using Vβ primer sets 1-20 and Cβ primer, the sequences of which are shown in Table 1. The reaction mixture for PCR amplification consisted of 0.4 μl cDNA, 0.4 μl Cβ primers (25 mM), 1.6 μl dNTP (2.5 mM), and 1 U Taq polymerase (Takara Biotechnology). Amplification proceeded for 35 cycles in a Perkin-Elmer Cetus thermocycler (Perkin-Elmer, Norwalk, USA) under the following, protocol: denaturation at 94°C for 1 min, annealing at 60°C for 2 min, and primer extension at 72°C for 3 min.

Table 1. Sequence of primers for each Vβ repertoire used for polynerase chain reaction (PCR)

Primer	5′→3′ sequence
Vβ1	GCACAACAGTTCCCTGACTTGCAC
Vβ2	TCATCAACCATGCAAGCCTGACCT
Vβ3	GTCTCTAGAGAGAAGAAGGAGCGC
Vβ4	ACATATGAGAGTGGATTTGTCATT
Vβ5.1	ATACTTCAGTGAGACACAGAGAAAC
Vβ5.2–3	TTCCCTAACTATAGCTCTGAGCTG
Vβ6.1–3	AGGCCTGAGGGATCCGTCTC
Vβ7	CCTGAATGCCCCAACAGVTCTC
Vβ8	ATTTACTTTAACAACAACGTTCCG
Vβ9	CCTAAATCTCCAGACAAAGCTCAC
Vβ10	CTCCAAAAACTCATCCTGTACCTT
Vβ11	TCAACAGTCTCCAGAATAAGGACG
Vβ12	AAAGGAGAAGTCTCAGAT
Vβ13.1	CAAGGAGAAGTCCCCAAT
Vβ13.2	GGTGAGGGTACAACTGCC
Vβ14	GTCTCTCGAAAAGAGAAGAGGAAT
Vβ15	AGTGTCTCTCGACAGGCACAGGCT
Vβ16	AAAGAGTCTAAACAGGATGAGTCC
Vβ17	CAGATAGTAAATGACTTTCAG
Vβ18	GATGAGTCAGGAATGCCAAAGGAA
Vβ19	CAATGVVVVAAGAACGCACCCTGC
Vβ20	AGCTCTGAGGTGCCCCAGAATCTC
3′Cβ	TTCTGATGGCTCAAACAC
5′Cα	GAACCCTGACCCTGCCCGTGTACC
3′Cα	ATCATAAATTCGGGTAGGATCC

Single-strand conformation polymorphism analysis

Analysis of T-cell clonality was performed using the SSCP method described by Yamamoto et al.,[11] with minor modifications. Briefly, the amplified cDNA was diluted 1:9 with a denaturing solution (95% formamide, 10 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) and heated at 95°C for 3 min.

Two μl of the heated sample was electrophoresed in a non-denaturing 5% polyacrylamide gel containing 10% glycerol at 35 W constant power for approximately 2.5 h. The denatured cDNA in the polyacrylamide gel was transferred to a positively charged nylon filter membrane (Hybond-N+; Amersham International Buckinghamshire, UK) by electrophoresis at a constant current of 200 mA for approximately 1 h. The filter was then hybridized with a ^{32}P-labeled internal Cβ1 and Cβ2 oligonucleotide probe mixture (5′-GAG GAC CTG AAA/G AAG/C GTG TTC CCA CCC-3′). Hybridization was performed at 60°C overnight in 5 × Denhardt's solution (Ficoll, borine serum albumin [BSA]: polyvinyl pyrolidone 1:1:1), 4 × SSC (NaCl, sodium citrate 3 mixture), 0.1% SDS, 10 mM EDTA, 40 mM Tris-HCl, and sonicated salmon sperm DNA. The filter was washed at low stringency, i.e., with 1 × SSC containing 0.1% SDS, and then exposed to an autoradiogram imaging screen, followed by analysis using a Bioimage Analyzing System (BAS 1000; Fuji Film, Tokyo, Japan). Each sharply stained band was counted as a single band.

Statistical analysis

Data values are expressed as means ± SD. The optimum concentration of PDC that resulted in the maximal PBMC response was determined by Kruskal-Wallis's test. Comparisons were performed with Mann-Whitney's *U*-test. A two-tailed *P* value of <0.05 was considered significant.

Results

Maximum response of peripheral blood mononuclear cells to PDC

The mean number of incorporated counts per min (cpm) of PBMCs without PDC stimulation was similar in the healthy controls (1557 ± 696) and PBC patients (1207 ± 540). The SI dose response curves of PDC (3-3 × 10^{-4} μg/ml)-stimulated PBMCs isolated from six PBC patients and six healthy controls showed that the optimal PDC concentration for PBMC stimulation in PBC patients was 3 × 10^{-1} μg/ml (Fig. 2). The SIs of the 12 PBC patients whose PBMCs were stimulated with 3 × 10^{-1} μg/ml of PDC are shown in Fig. 3, along with the SIs for the 6 healthy controls. The mean SI (2.15) for the 12 patients was significantly higher ($P < 0.01$) than that (1.05) for the healthy controls. When an SI of more than the mean ± 2SD of the healthy controls was considered to be indicative of stimulation, 10 of the 12 PBC patients showed positive stimulation.

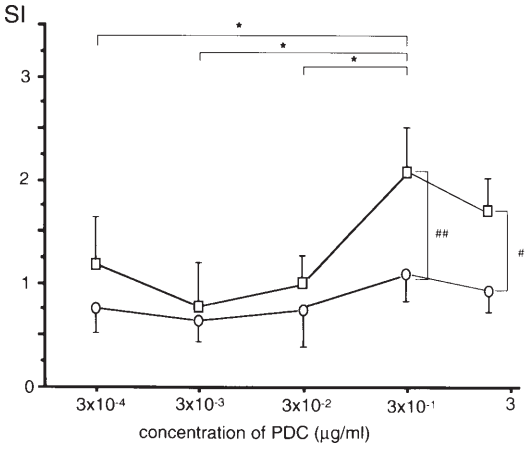

Fig. 2. Dose response curve of PDC-stimulated peripheral blood mononuclear cells (PBMCs) isolated from patients with primary biliary cirrhosis (PBC; *squares*; $n = 6$) and healthy controls (*circles*; $n = 6$). The highest stimulation indexes (SIs) were obtained at a PDC concentration of 3 × 10^{-1} μg/ml for the PBMCs of PBC patients. At a PDC concentration of 3 × 10^{-1} μg/ml, the mean SI for the patients (2.15 ± 0.36) was significantly ($P < 0.01$) higher than that for the healthy controls (1.05 ± 0.15). *$P < 0.01$ (Kruskal-Wallis test); #$P < 0.05$; ##$P < 0.01$ (Mann-Whitney *U*-test)

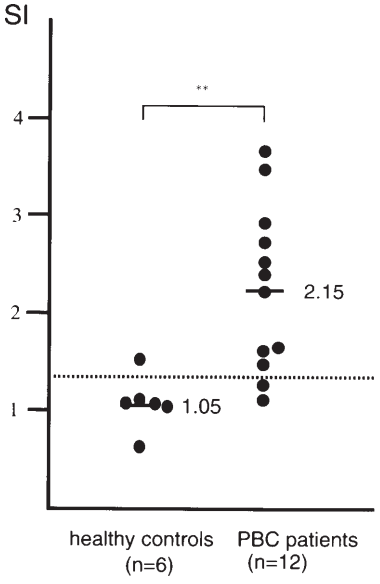

Fig. 3. SIs for PDC (3 × 10^{-1} μg/ml)-stimulated PBMCs isolated from the 12 PBC patients and 6 healthy controls. The mean SI for PBMCs of the 12 patients (2.15) was significantly ($P < 0.01$) higher than that for the healthy controls (1.05). When an SI of more than 1.35 (mean + 2SD of healthy controls) was considered to be indicative of stimulation, 10 of the 12 PBC patients exhibited positive stimulation. **$P < 0.01$ Mann-Whitney *U*-test

Analysis of clonal accumulation of T cells from PBC patients and healthy controls

The TCR Vβ repertoires of PDC-stimulated PBMCs, unstimulated PBMCs, and liver biopsy specimens were determined by SSCP analysis.

Representative results for TCR Vβ repertoires 2, 4, 10, 12, 16, and 18 of PBC patient no. 12 and of one of the healthy controls are shown in Fig. 4a,b, respectively. Several different T-cell clones, some of which showed the same mobility, were evident in both the PDC+ and PDC− samples, as demonstrated by SSCP analysis. In the corresponding lanes for the healthy control, only smearing was observed and no distinct T-cell population could be identified.

The TCR Vβ repertoires determined for the 12 PBC patients and 6 healthy controls are summarized in Table 2. For the 12 patients, the average number of bands from Vβ 1 to Vβ 20 was significantly ($P < 0.01$) smaller for PDC-stimulated PBMCs (36.8) than unstimulated PBMCs (62.0). No marked tendency towards the use of specific Vβs was noted, although frequent use of Vβ 6,7, and 13.2 was observed. Figure 5a–c shows representative data for Vβ 12, obtained from patient no. 7 (Fig. 5a), Vβ 9 from patient no. 10 (Fig. 5b), and Vβ 13.1 from patient no. 11 (Fig. 5c). For all three patients, the PDC-stimulated PBMCs had fewer T-cell clones than the unstimulated PBMCs [two versus four SSCP bands for patient no. 7 (Fig. 5a), two versus five for patient no. 10 (Fig. 5b), and two versus five for patient no. 11 (Fig. 5c)].

Amplification of five PBC patient liver samples resulted in a total of 26 bands, ranging from one in patient no. 1 to seven in patient no. 4. The mobility of 23 of these 26 T-cell clone bands was virtually identical to that of the corresponding PBMC bands. Figure 6a–c shows representative data for Vβ 8 obtained from

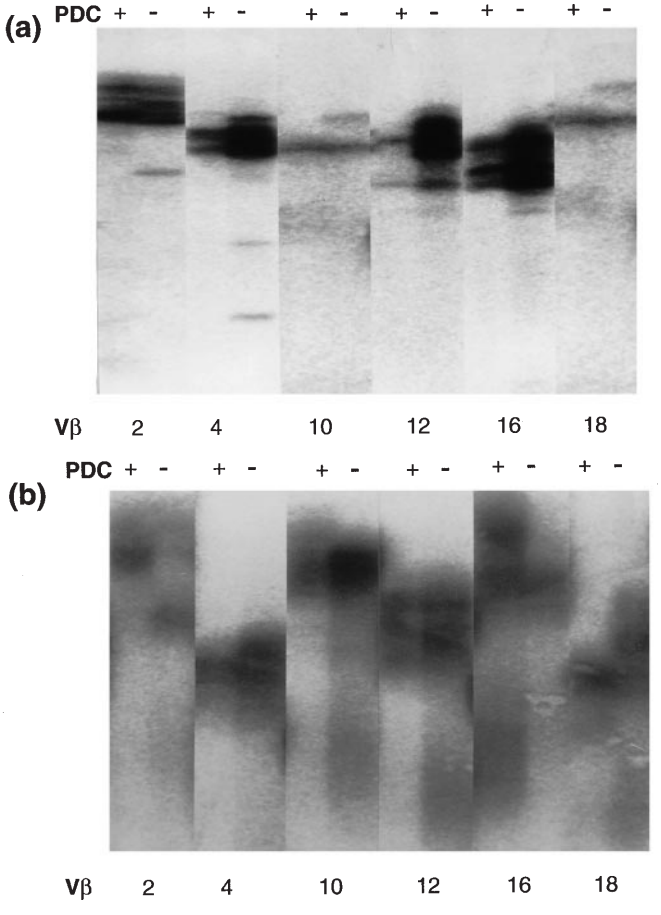

Fig. 4a,b. Representative results of single-strand conformation polymorphism (SSCP) analysis of T-cell receptors (TCR) Vβ 2, 4, 10, 12, 16, and 18 messages expressed **a** in PBMCs of patient no. 12 and **b** the PBMCs of one of the healthy controls. *PDC+* and *PDC− lanes* indicate PDC-stimulated and unstimulated PBMCs, respectively. More bands representing T-cell clones were noted in the unstimulated PBMC samples than in the stimulated PBMC sample of patient no. 12 (**a**). No distinct populations of T-cell clones were observed for either the PDC-stimulated or unstimulated PBMCs of the healthy control (**b**)

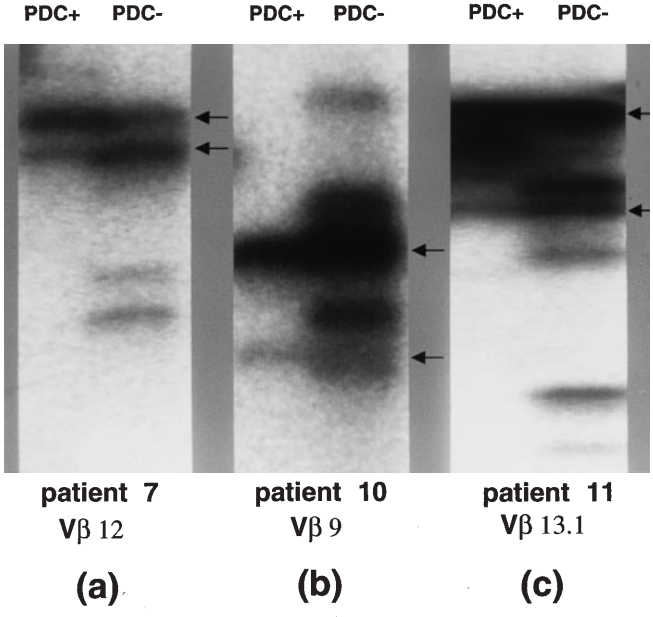

Fig. 5a,b,c. Comparison of results of SSCP analysis of TCR Vβ repertoires of PDC-stimulated and unstimulated PBMCs. Results for **a** Vβ 12 of patient no. 7, **b** Vβ 9 of patient no. 10, and **c** Vβ 13.1 of patient no. 11 are shown. For all three patients, the number of T-cell clones in PDC-stimulated PBMCs was lower than that in unstimulated PBMCs; two versus four bands for patient no. 7 (**a**), two versus five for patient no. 10 (**b**), and two versus five for patient no. 11 (**c**). *Arrows* indicate bands common to both PDC-stimulated and unstimulated PBMCs

Table 2. SSCP analysis of T-cell population with each Vβ repertoire in peripheral blood mononuclear cells (PBMCs) stimulated with pyruvate dehydrogenase complex (PDC+) or unstimulated with PDC (PDC−) of 12 PBC patients and 6 healthy controls, along with unstimulated liver tissues of 5 PBC patients

		No. of distinct bands in Vβ																						
PBC patients		1	2	3	4	5.1	5.2–3	6.1–3	7	8	9	10	11	12	13.1	13.2	14	15	16	17	18	19	20	Total
1	PDC+	1	1	2	S	2	S	4	3	2	4	1	2	3	3	4	1	3	1	3	2	4	5	51
	PDC−	1	4	3	S	3	S	5	6	2	5	2	3	5	S	6	1	4	5	4	4	7	7	77
	LT	N	N	N	N	N	N	N	N	2(2)	N	N	N	N	N	N	N	N	N	N	N	N	N	2(2)
2	PDC+	2	3	N	2	S	4	2	3	2	2	N	1	2	S	1	3	1	1	1	2	3	3	38
	PDC−	4	5	6	4	S	5	5	4	4	3	S	5	5	1	3	8	2	3	1	2	4	6	80
	LT	N	N	N	N	N	N	2(2)	1(1)	N	N	N	N	N	N	N	N	N	N	N	N	N	N	3(3)
3	PDC+	2	2	1	2	3	2	4	4	3	3	2	2	1	4	4	3	2	2	2	2	2	2	54
	PDC−	2	4	1	2	4	4	6	7	3	5	5	N	4	6	4	6	4	4	5	4	2	1	83
	LT	N	N	1(1)	2(2)	1(1)	N	N	N	N	N	2(2)	N	N	N	N	N	2(2)	N	N	1(1)	N	1(1)	10(10)
4	PDC+	3	1	1	N	1	1	1	N	1	N	2	N	2	1	1	N	1	2	2	S	1	2	23
	PDC−	3	1	1	N	2	1	1	N	4	N	1	S	3	1	1	N	2	5	4	S	1	2	33
	LT	1	N	2(1)	N	N	N	2(1)	N	N	N	N	N	N	1(1)	N	N	N	N	N	N	2(1)	N	8(5)
5	PDC+	2	S	1	2	1	2	3	2	1	S	2	1	2	1	S	2	1	1	2	1	S	N	27
	PDC−	3	S	3	4	3	2	2	4	3	S	4	2	3	2	S	4	3	1	3	3	S	N	49
	LT	N	N	N	N	1(1)	N	N	N	1(1)	S	N	N	N	N	N	N	N	1(1)	N	N	N	N	3(3)
6	PDC+	1	3	S	S	3	S	3	2	2	2	3	2	2	2	3	2	2	N	S	S	6	4	42
	PDC−	3	2	S	S	6	S	4	5	2	2	1	2	2	2	4	4	N	S	S	S	5	5	49
7	PDC+	1	2	S	1	2	N	1	N	1	2	2	N	2	3	3	2	5	N	N	1	4	2	34
	PDC−	N	3	S	2	2	N	3	7	N	3	N	N	4	3	6	1	N	N	2	3	4	1	44
8	PDC+	1	2	N	2	3	N	3	N	1	3	1	1	N	2	2	N	1	2	1	2	2	3	32
	PDC−	2	3	N	2	3	N	4	5	3	3	1	5	S	3	3	1	1	2	1	6	5	2	55
9	PDC+	2	1	2	1	4	N	1	2	2	1	3	2	1	2	2	4	S	S	2	2	1	3	38
	PDC−	1	2	4	4	6	S	3	2	5	4	6	1	2	4	5	4	2	1	2	4	2	5	69
10	PDC+	N	N	1	N	2	N	N	2	1	2	2	1	N	1	2	2	1	N	N	N	N	1	18
	PDC−	3	5	2	N	4	3	4	5	3	5	5	3	4	4	1	3	3	2	3	4	2	2	70
11	PDC+	1	N	2	2	S	1	3	4	1	2	N	1	2	2	3	N	1	2	3	1	1	2	34
	PDC−	2	N	3	3	S	2	4	5	3	4	N	2	4	5	6	N	3	4	3	2	4	3	62
12	PDC+	N	3	4	2	1	1	2	N	3	1	1	3	2	3	6	2	6	3	4	1	3	N	51
	PDC−	N	5	3	6	2	2	6	N	4	3	2	5	4	6	6	4	6	4	4	2	1	N	75
Total	PDC+	16	18	14	14	22	11	27	27	20	22	19	16	19	24	31	21	24	14	20	14	27	27	
	PDC−	24	34	23	27	35	19	47	50	36	37	27	28	40	37	45	36	25	31	32	34	37	34	
	LT	1(1)	0	3(2)	2(2)	2(2)	0	4(3)	1(1)	3(3)	0	2(2)	0	0	1(1)	0	0	2(2)	1(1)	0	1(1)	2(1)	1(1)	

		No. of distinct bands in Vβ																						
Six healthy controls		1	2	3	4	5.1	5.2–3	6.1–3	7	8	9	10	11	12	13.1	13.2	14	15	16	17	18	19	20	Total
Total	PDC+	1	1	1	2	S	1	2	S	S	1	2	2	4	2	2	1	1	2	3	1	2	1	
	PDC−	2	2	1	1	S	2	1	S	S	2	3	2	S	S	S	1	1	2	2	2	1	S	

S, Smear (no distinct bands); N, no PCR product; LT, liver tissue; SSCP, single-strand conformation polymorphism
Figures in parentheses are the number of common usage of TCR clonality in both PBMCs and the liver infiltrating cells

patient no. 1 (Fig. 6a), Vβ 10 from patient no. 3 (Fig. 6b), and Vβ 3 from patient no. 4 (Fig. 6c). The mobility of the liver T-cell clones was identical to that of both the stimulated and unstimulated PBMCs.

For the six healthy controls, smearing was observed in both PDC+ and PDC− lanes for almost all Vβs, as shown in Fig. 4b.

Discussion

The data presented here reveal a greater variety of T-cell clones in samples of unstimulated PBMCs isolated from PBC patients compared with findings in the healthy controls. Presumably, these T-cell clones had already proliferated in vivo in response to certain ongoing stimuli. This hypothesis is supported by the finding that a number of clonotypic expansions were detected during antigenic stimulation in vivo in almost all of the Vβ families tested.[22] A few T-cell clones were observed in the PBMCs of the healthy controls; these may have been remnants of earlier expansions in vivo as a result of previous exposure to antigen.

The average of the total number of T-cell clones from Vβ 1 to Vβ 20 in PDC-stimulated PBMCs of the 12 patients was less than that of the unstimulated PBMCs from these patients. This finding may indicate a deletion of T-cell clones which had been sensitized to PDC as a

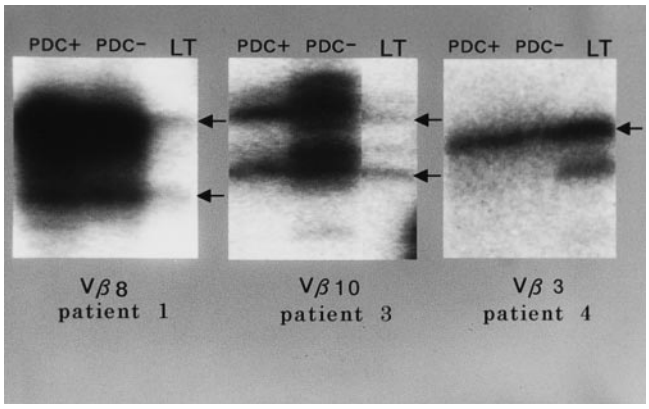

Fig. 6a,b,c. Comparison of results of SSCP analysis of TCR Vβ repertoires of PDC-stimulated and unstimulated PBMCs and of liver biopsy tissue for **a** Vβ 8 of patient no. 1, **b** Vβ 10 of patient no. 3, and **c** Vβ 3 of patient no. 4. Two T-cell clone populations in the Vβ 8 of the liver tissue of patient no. 1 showed the same mobility as two bands from both stimulated and unstimulated PBMCs (**a**). Similarly, Two T-cell clone populations of the Vβ 10 obtained from the liver tissue of patient no. 3 showed the same mobility as two bands obtained from the stimulated PBMCs and two of five bands from the unstimulated PBMCs (**b**). With Vβ 3 from the liver tissue of patient no. 4, the upper clone obtained from the liver tissue showed the same mobility as a band from both stimulated and unstimulated PBMCs (**c**)

result of a previous PDC stimulation. Such a deletion has been reported to result from activation-induced cell death through apoptosis.[23,24] However, the present study provides no evidence that the reduced number of T-cell clones in PDC-stimulated PBMC is due to antigen-induced apoptosis.

On the other hand, PDC-specific T cells may be preferentially expanded and thus, only clonal bands specific for PDC-specific T cells are detected. In addition, in patient no. 4, the TCR clonality of the liver-infiltrating cells was greater than that of PBMCs, indicating that T cells in the liver of PBC patients may be stimulated by other antigens in addition to PDC. Moreover, the lack of a biased pattern of Vβ expression in PBMCs of patients with PBC indicates that superantigenic stimulation does not occur in these patients. A number of factors could have influenced the TCR repertoire in the blood during this time, such as continued autoimmune stimulation related to PBC, unrelated infections, or other immunological events, thereby explaining the heterogeneous Vβ expression seen in the PBMCs of these PBC.

In the present study, the T-cell clones in the PBMCs stimulated with PDC and liver-infiltrated lymphocytes exhibited very similar mobility on SSCP analysis. Because DNA amplified from the same clone migrates to the identical position on SSCP analysis,[11,25] and because bands that migrate to the same position on SSCP analysis have the same amino acid sequence,[12] these data suggest that common clonal accumulation occurs in both PBMCs and infiltrated T cells in the liver. Further research will focus on whether these oligoclonal T cells possess cytotoxic activity against bile duct epithelial cells and whether they contribute to the pathogenesis of PBC.

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