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### Research Article

Short-term culture of peripheral blood mononuclear cells (PBMC) derived from patients with human T cell lymphotropic virus type I-associated myelopathy (HAM)/tropical spastic paraparesis resulted in dominance by DR+ activated CD8+ T cells. Variations in the T cell receptor (TCR) V alpha and V beta chains in these cells were analyzed, and in all 10 patients examined, 2-3 V gene families were dominant in both TCR V alpha and V beta. In five patients we examined, cultured lymphocytes contained cytotoxic lymphocytes for p40tax (patients HAM2, 3, 7, and 8) or env protein (patient HAM4) of human T lymphotropic virus type I. In patients HAM2 and HAM8, cultured lymphocytes contained a large proportion of V beta 8+ CD8+ and/or V beta 12+ CD8+ cells. The sequence of V beta 8+ and V beta 12+ cDNA revealed that they were oligoclonal with identical or similar sequences in each patient. Elimination experiments with monoclonal antibodies for TCR V beta 8 and V beta 12 showed that they were CD8+ cytotoxic T lymphocytes (CTL) for p40tax. In addition, flow cytometry and sequencing analysis of uncultured PBMC revealed that in HAM2, V beta 8+ CTL and their precursors account for 7% and V beta 12+ CTL and their precursors account for 18% of total CD8+ cells. This indicates the presence of two markedly expanded clones in [...]

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# Clonal Expansion of CD8<sup>+</sup> Cytotoxic T Lymphocytes against Human T Cell Lymphotropic Virus Type I (HTLV-I) Genome Products in HTLV-I-associated Myelopathy/Tropical Spastic Paraparesis Patients

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## Abstract

Short-term culture of peripheral blood mononuclear cells (PBMC) derived from patients with human T cell lymphotropic virus type I-associated myelopathy (HAM)/tropical spastic paraparesis resulted in dominance by DR<sup>+</sup> activated CD8<sup>+</sup> T cells. Variations in the T cell receptor (TCR) V $\alpha$  and V $\beta$  chains in these cells were analyzed, and in all 10 patients examined, 2–3 V gene families were dominant in both TCR V $\alpha$  and V $\beta$ . In five patients we examined, cultured lymphocytes contained cytotoxic lymphocytes for p40<sup>tax</sup> (patients HAM2, 3, 7, and 8) or env protein (patient HAM4) of human T lymphotropic virus type I. In patients HAM2 and HAM8, cultured lymphocytes contained a large proportion of V $\beta$ 8<sup>+</sup> CD8<sup>+</sup> and/or V $\beta$ 12<sup>+</sup> CD8<sup>+</sup> cells. The sequence of V $\beta$ 8<sup>+</sup> and V $\beta$ 12<sup>+</sup> cDNA revealed that they were oligoclonal with identical or similar sequences in each patient. Elimination experiments with monoclonal antibodies for TCR V $\beta$ 8 and V $\beta$ 12 showed that they were CD8<sup>+</sup> cytotoxic T lymphocytes (CTL) for p40<sup>tax</sup>. In addition, flow cytometry and sequencing analysis of uncultured PBMC revealed that in HAM2, V $\beta$ 8<sup>+</sup> CTL and their precursors account for 7% and V $\beta$ 12<sup>+</sup> CTL and their precursors account for 18% of total CD8<sup>+</sup> cells. This indicates the presence of two markedly expanded clones in vivo. No common dominant TCR V $\alpha$  or V $\beta$  were observed among 10 HAM patients analyzed. (*J. Clin. Invest.* 1994. 94:1830–1839.) Key words: T cell receptor • V $\beta$  gene • tax protein • cell-mediated cytotoxicity • polymerase chain reaction

## Introduction

CD8<sup>+</sup> cytotoxic T lymphocytes (CTL)<sup>1</sup> are widely accepted as being the primary effectors against virus-infected cells. They

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1. Abbreviations used in this paper: CTL, cytotoxic T lymphocytes; EB-B, EBV-transformed B cell line; HAM, HTLV-I-associated myelopathy; HTLV-I, human T cell lymphotropic virus type I; PE, phycoerythrin; r, recombinant; RT-PCR, reverse transcription-polymerase chain reaction; TCR, T cell receptor.

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specifically recognize the peptides encoded by viral genomes in the context of MHC class I molecules, leading to the destruction of virus-infected cells. The virus-specific CTL precursor frequencies in these viral infections have been quantitated by limiting dilution assays, ranging from 2–100/10<sup>6</sup> peripheral blood mononuclear cells (PBMC) in Epstein Barr virus (EBV), varicella-zoster virus, and human cytomegalovirus (1–3). Relatively higher frequencies of up to 488/10<sup>6</sup> PBMC were observed in human immunodeficiency virus type I depending upon the target molecules and disease status (4).

The presence of virus-specific T cells and their precursors has also been identified in individuals infected with human T lymphotropic virus type I (HTLV-I), which is closely associated with adult T cell leukemia (5–7) and HTLV-I-associated myelopathy/tropical spastic paraparesis (abbreviated as HAM [HTLV-I-associated myelopathy] throughout this manuscript) (8–11). In particular, in patients with HAM, dominant humoral as well as cellular responses against HTLV-I were commonly observed (12–14). In the recent report, HTLV-I-specific CTL precursor frequencies in HAM patients have ranged between 1/75–1/320 PBMC, 40–280-fold higher than those in asymptomatic HTLV-I-infected individuals (15).

Here we report our analyses of the T cell receptor (TCR) V gene and clonality of CD8<sup>+</sup> T cells derived from HAM patients cultured for a short term in the absence of exogenous antigen stimulation. These CD8<sup>+</sup> cells are likely oligoclonal CTL specific for HTLV-I. The possible mechanism that results in the dominance of virus-specific CTL clones is also discussed.

## Methods

**Cells and cell cultures.** PBMC from HAM patients and normal individuals were isolated from heparinized venous blood by Ficoll-Conray density gradient centrifugation and cultured in RPMI 1640 with 20% fetal calf serum (FCS) and 400 IU/ml recombinant IL-2 (rIL-2) (Takeda Pharmaceutical Co., Osaka, Japan). B lymphoblastoid cell lines (EB-B cells) were established by the standard EBV transformation of PBMC (16).

**Antibodies.** The mAbs were as follows: anti-CD4 mAb, OKT4; anti-CD8 mAb, OKT8; anti-CD3 mAb, OKT3 (American Type Culture Collection, Rockville, MD); anti-MHC class II DR mAb, NL-12 (provided by Dr. R. Ueda, Aichi Cancer Center Institute, Nagoya, Japan); R-phycoerythrin (PE)-conjugated anti-human CD4 mAb, DAKO-CD4 (DAKOPATTS, Copenhagen, Denmark); R-PE-conjugated anti-human CD8 mAb, DAKO-CD8 (DAKOPATTS); anti-TCR V $\beta$ 5 mAb, Diversi-T $\beta$ V5(a) and (b); anti-TCR V $\beta$ 6 mAb, Diversi-T $\beta$ V6(a); anti-TCR V $\beta$ 8 mAb, Diversi-T $\beta$ V8(a); anti-TCR V $\beta$ 12 mAb, Diversi-T $\beta$ V12(a) (all anti-TCR mAbs were purchased from T Cell Sciences, Inc., Cambridge, MA).

**RNA extraction, reverse transcription-polymerase chain reaction (RT-PCR), and Southern blots.** Total RNA was prepared according to Chomczynski and Sacchi (17). The cDNAs were synthesized with an

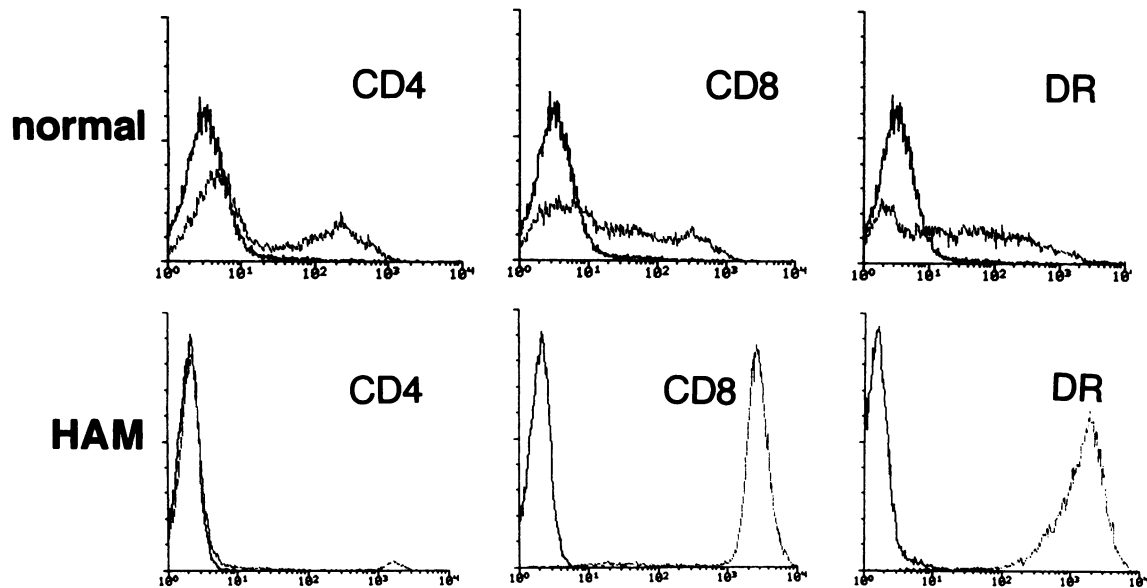


Figure 1. The surface phenotype of cultured lymphocytes. PBMC derived from a normal individual or a patient (HAM1) were cultured for 2 wk, then stained with the mAbs DAKO-CD4, DAKO-CD8, and NL-12 (DR). For NL-12, FITC-conjugated goat anti-mouse IgG was used as a second antibody. Samples were analyzed on a FACS<sup>®</sup>, and the results are expressed as fluorescence intensity versus the number of cells.

A

primer	5' → 3' sequence	primer	5' → 3' sequence
V $\alpha$ 1	TTGCCCTGAGAGATGCCAGAG	V $\beta$ 1	GCACAACAGTTCCTGACTTGCAC
V $\alpha$ 2	GTGTTCCAGAGGGAGCCATTGCC	V $\beta$ 2	TCATCAACCATGCAAGCCTGACCT
V $\alpha$ 3	GGTGAACAGTCAACAGGGAGA	V $\beta$ 3	GTCTCTAGAGAGAGAAGGAGCGC
V $\alpha$ 4	ACAAGCATTACTGTACTCCTA	V $\beta$ 4	ACATATGAGAGTGGATTGTGATT
V $\alpha$ 5	GGCCCTGAACATTCCAGGA	V $\beta$ 5.1	ATACTTCAGTGAGACACAGAGAAG
V $\alpha$ 6	GTCACCTTCTAGCCTGCTGA	V $\beta$ 5.2-3	TTCCCTAACTATAGCTCTGAGCTG
V $\alpha$ 7	AGGAGCCATTGTCCAGATAAA	V $\beta$ 6.1-3	AGGCCTGAGGGATCCGCTCTC
V $\alpha$ 8	GGAGAGAATGTGGAGCAGCATC	V $\beta$ 7	CCTGAATGCCCAACAGCTCTC
V $\alpha$ 9	ATCTCAGAGCTTGTGATAATA	V $\beta$ 8	ATTTACTTTTAAACAACAGTTCGG
V $\alpha$ 10	ACCCAGCTGCTGGAGCAGAGCCCT	V $\beta$ 9	CCTAAATCTCCAGACAAAGCTCAC
V $\alpha$ 11	AGAAAAGCAAGGCAAGTGT	V $\beta$ 10	CTCCAAAACTCATCTGTACCTT
V $\alpha$ 12	CAGAAGGTAAGTCAAGCGCAGACT	V $\beta$ 11	TCAACAGTCTCCAGAATAAGGAGC
V $\alpha$ 13	GCTTATGAGAACTGCGT	V $\beta$ 12	AAAGGAGAAGTCTCAGAT
V $\alpha$ 14	GCAGCTTCCCTCCAGCAAT	V $\beta$ 13.1	CAAGGAGAAGTCCCAAT
V $\alpha$ 15	AGAACCCTGACTGCCAGGAA	V $\beta$ 13.2	GGTGAGGGTACAACCTGCC
V $\alpha$ 16	CACTCTCCATGGACTCATATGA	V $\beta$ 14	GTCTCTCGAAAAGAGAGAGGAAT
V $\alpha$ 17	GACTATACTAACAGCATGT	V $\beta$ 15	AGTGTCTCTCGACAGCCAGGCT
V $\alpha$ 18	AGGCTCAAGTTCAGGTGTCAG	V $\beta$ 16	AAAGAGTCTAAACAGGATGATCC
3' C $\alpha$	AATAGGCAGACAGACTTGTCACTGGA	V $\beta$ 17	CAGATAGTAAATGACTTTTCAG
probe		V $\beta$ 18	GATGAGTCAAGAAATGCCAAAGGAA
CA-2	GATATCCAGAACCCTGACCCCT	V $\beta$ 19	CAATGCCCAAGAACGCCACCCCTGC
		V $\beta$ 20	AGCTCTGAGGTGCCCCAGAATCTC
		3' C $\beta$	TTCTGATGGCTCAAACAC
		probe	
		CB-2	GAGGACCTGAACAAGGTGTTCACCCGAG

B

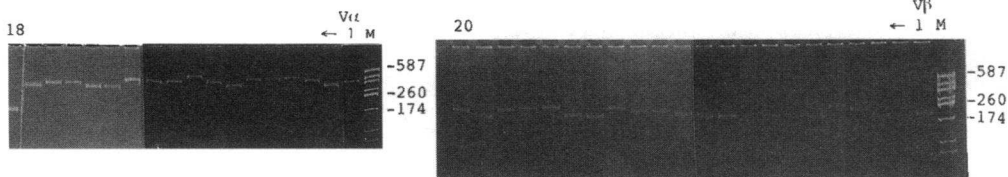


Figure 2. Sequences of the TCR primers used for PCR. A shows sequences of primers and probes. The primers used for PCR were as previously published (28, 29). The size of amplified V $\alpha$  bands with V $\alpha$  and 3' C $\alpha$  or V $\beta$  bands with V $\beta$  and 3' C $\beta$  primers ranged from ~ 300 to 400 bp (V $\alpha$ 18 is ~ 170 bp) or 170 to 220 bp, respectively. CA-2 and CB-2 were used as oligonucleotide probes that located within TCR C $\alpha$  or C $\beta$  region for Southern blotting after <sup>32</sup>P-labeling. B shows ethidium bromide staining of the amplified products of cultured PBMC from a normal individual. Products after 24 cycles of PCR with the 5' V $\alpha$  and 3' C $\alpha$  primers or the 5' V $\beta$  and 3' C $\beta$  primers were separated on a 12% acrylamide gel. The right contains the molecular weight marker (M), HaeIII-digested pUC19 plasmid.

Table I. Phenotypes of Cultured Lymphocytes from HAM Patients and Normal Individuals

PBMC donors	Percentage of positive cells		
	CD4 <sup>+</sup>	CD8 <sup>+</sup>	DR <sup>+</sup>
<b>HAM patients</b>			
1	3	88	91
2	2	73	92
3	7	78	87
4	11	78	81
5	6	89	82
6	12	80	79
7	10	84	75
8	5	90	92
9	14	80	74
10	9	78	79
<b>Normal individuals</b>			
1	52	40	23
2	38	41	24
3	48	40	20

PBMC from HAM patients and normal individuals were cultured for 2 wk in RPMI 1640 with 20% FCS and 400 IU/ml of rIL-2, and then were stained with anti-CD4, anti-CD8, or anti-MHC class II DR mAb, followed by FITC-conjugated goat anti-mouse IgG. Samples were analyzed and the percent value for each phenotype was calculated using a FACS®.

oligo-dT<sub>14</sub> primer and PCR was performed as described (18, 19). A combination of TCR V $\alpha$  or V $\beta$  primer as a 5'-primer and TCR C $\alpha$  or C $\beta$  primer as a 3'-primer was used (see Fig. 2). After 22 cycles of PCR, all TCR V $\alpha$  and V $\beta$  gene families were detectable in samples from normal volunteers.

The amplified products separated by PAGE were transferred onto nylon filters (Gene Screen Plus; DuPont, Boston, MA). Prehybridization and hybridization with oligonucleotide probes labeled with [<sup>32</sup>P] $\gamma$ -ATP proceeded at 43°C for 4 and 15 h, respectively, and the membrane was exposed to an imaging plate (BAS-III; Fuji Photo Film Co., Tokyo, Japan) for 4 h. The radioactivity of specific bands was measured by the Bio-Imaging Analyzer (BAS 2000; Fuji Photo Film Co.).

**DNA sequencing.** To sequence the TCR genes, after 26 cycles of PCR using V $\beta$ 8 or V $\beta$ 12 primers as 5'-primers and the C $\beta$  3'-primer, PCR products were cloned into pT7Blue(R)T-Vector (Novagen, Inc., Madison, WI). Cloned inserts were sequenced using the Taq Dye Primer Cycle Sequencing kit (Applied Biosystems, Inc., Foster City, CA) with the DNA sequencer (model 370A; Applied Biosystems, Inc.).

**Preparation of autologous EB-B cells infected with recombinant vaccinia viruses.** HTLV-I genome was selectively expressed (20–23) in the following recombinant vaccinia viruses: LO5-gag for expression of p19, p24, and p15; LO5-env for expression of gp62, gp46, and gp21; LO5-40x for expression of p40<sup>tax</sup>; and LO5-HA<sup>-</sup> as a vector control. Details of the HTLV-I/vaccinia recombinants have been described elsewhere (20–22). EB-B cells were infected with vaccinia recombinants for 36–40 h, then the antigen expression was checked using mAb REY-7 (rat IgG) (24) for env protein, mAb ATLVI11 (a gift from Dr. R. Ueda) for gag protein, and mAb Lt4 (25) for p40<sup>tax</sup> protein.

**Cell-mediated cytotoxicity assay.** The method has been described previously (26). Cells labeled with Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> (1 × 10<sup>4</sup>/100  $\mu$ l) were incubated with a serially diluted effector cell suspension (100  $\mu$ l) in 96-well round-bottomed plates for 4 h at 37°C in 5% CO<sub>2</sub>. The percentage

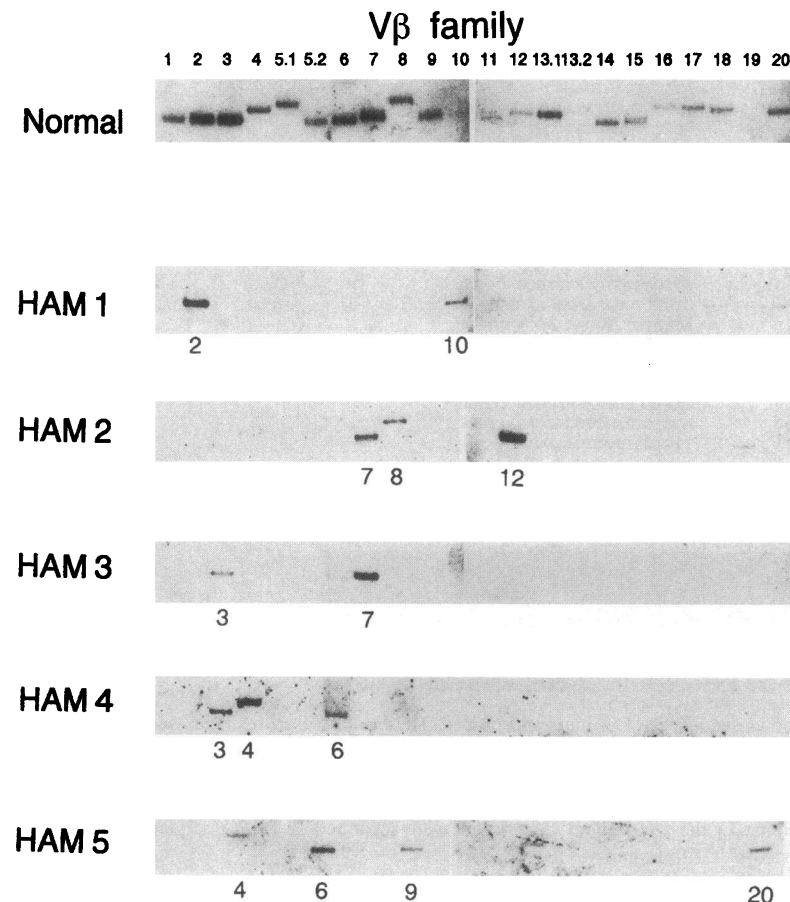


Figure 3. Southern blot of the RT-PCR products of the TCR V $\beta$  family. After 22 cycles of amplification, RT-PCR products of TCR V $\beta$  families derived from cultured PBMC of normal individuals and HAM patients were Southern blotted and then hybridized with the C $\beta$  probe (CB-2). The results of a normal individual and five HAM patients are shown.

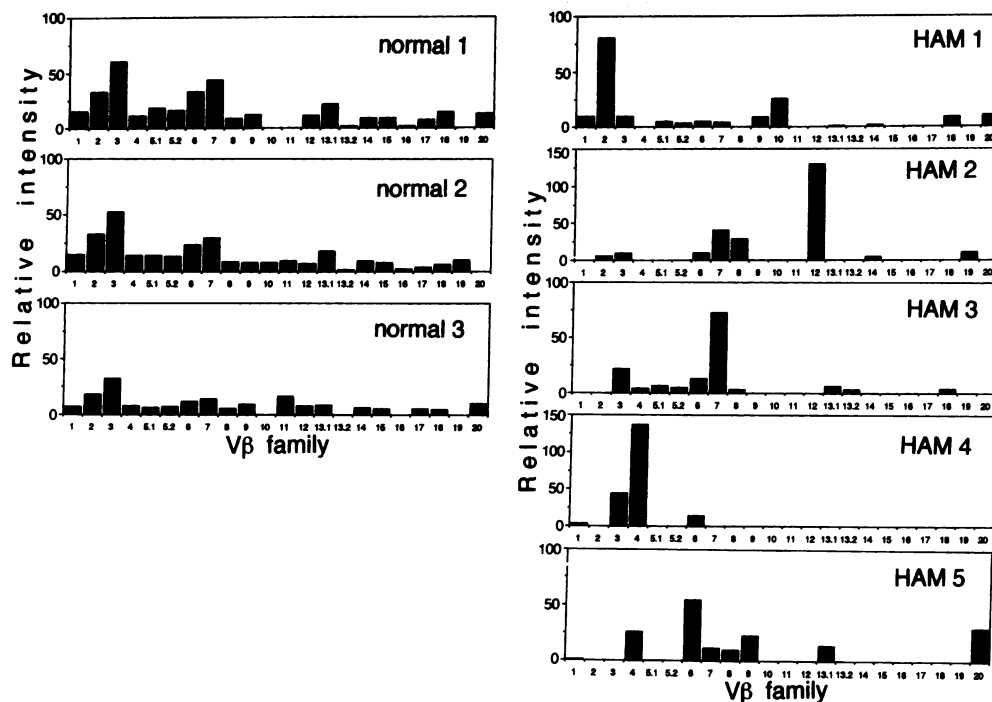


Figure 4. The relative intensity of bands of RT-PCR products of TCR V $\beta$  family. The intensity of each band shown in Fig. 3 was measured using a Bio-Imaging Analyzer. The number of the relative intensity corresponds to counts per minute in the Bio-Imaging Analyzer.

Table II. Nucleotide Sequences of TCRs Using V $\beta$ 8.1 and V $\beta$ 12.1 in Cultured PBMC from HAM2 and HAM8

TCR V $\beta$	Number of clones	Sequences			
		V	N D N	J	C
<b>HAM 2</b>					
V $\beta$ 8.1	10/10*	V $\beta$ 8.1...TGTGCCAGCATCCTAGGATGGGGG ...CysAlaSerIleLeuGlyTryGly		J $\beta$ 2.7	C $\beta$ 2
V $\beta$ 12.1	10/10	V $\beta$ 12.1...TGTGCCATCAGTGACTCAGGGGGC ...CysAlaIleSerAspSerGlyGly		J $\beta$ 1.1	C $\beta$ 1
<b>HAM 8</b>					
V $\beta$ 8.1	5/12	V $\beta$ 8.1...TGTGCCAGCAGTTGGGACATC ...CysAlaSerSerTrpAspIle		J $\beta$ 1.3	C $\beta$ 1
	4/12	V $\beta$ 8.1...TGTGCCAGCAGTTTTGATATC ...CysAlaSerSerPheAspIle		J $\beta$ 1.3	C $\beta$ 1
	1/12	V $\beta$ 8.1...TGTGCCAGAAGTCCCGACATC ...CysAlaArgSerProAspIle		J $\beta$ 1.3	C $\beta$ 1
	1/12	V $\beta$ 8.1...TGTGCCAGGAGCGGGACATC ...CysAlaArgSerAlaAspIle		J $\beta$ 1.3	C $\beta$ 1
	1/12	V $\beta$ 8.1...TGTGCCAGCAGTTCTGGGTGGGGG ...CysAlaSerSerSerGlyTrpGly		J $\beta$ 2.1	C $\beta$ 2

PBMC from HAM2 and HAM8 were cultured for 2 wk in RPMI 1640 with 20% FCS and 400 IU/ml of rIL-2, then total RNA was prepared. TCRs using V $\beta$ 8.1 and V $\beta$ 12.1 were amplified from cDNA by PCR with V $\beta$ 8.1 or V $\beta$ 12.1 and C $\beta$  primers. Multiple isolates from each cDNA were randomly selected and sequenced. The sequences of V $\beta$ 8.1, V $\beta$ 12.1, J $\beta$ , and C $\beta$  agreed with those reported previously (30, 31). \* Number of clones with sequences shown in the table/number of clones examined.

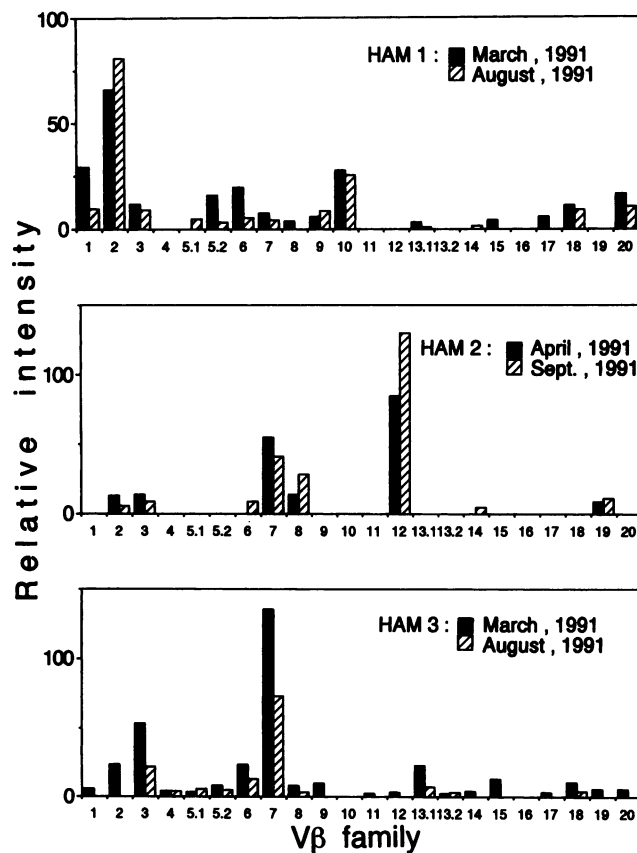


Figure 5. The similarity of shifted TCR V $\beta$  use in PBMC sequentially obtained from the same HAM patients. PBMC from three HAM patients were obtained on different occasions and RT-PCR was performed. The intensity of the bands on the PCR-Southern blot was measured using the Bio-Imaging Analyzer as shown in Fig. 4.

of specific lysis was calculated as follows:  $(a - b)/(c - b) \times 100$ ; where a is counts per minute in the supernatant of target cells mixed with effector cells; b is counts per minute in the supernatant of target cells incubated alone; and c is counts per minute after lysing the target cells with 1% of Nonidet P-40. To determine the phenotype of HTLV-I-specific CTL, elimination by panning using various mAbs was performed. Effector cells were incubated with mAbs for TCR V $\beta$ 8, TCR V $\beta$ 12, TCR V $\beta$ 6, CD4, CD8, or Lyt2.2 (murine CD8) independently on ice for 60 min. After washing twice, cells were plated on dishes coated with goat anti-mouse IgG (H + L) (Zymed Laboratories, Inc., South San Francisco, CA) at room temperature for 90 min according to the method of Wysocki and Sato (27). After incubation, nonadherent cells were collected and used as effector cells for the cell-mediated cytotoxicity assay as described above.

## Results

**Clonal expansion of CD8<sup>+</sup> cells in PBMC derived from HAM patients.** Short-term cultures (10–14 d) of PBMC derived from HAM patients in the presence of rIL-2 at 400 IU/ml resulted in the dominance of CD8<sup>+</sup> DR<sup>+</sup> T cells in all 10 samples studied. No CD8<sup>+</sup> T cell dominance was observed in PBMC derived from normal volunteers and cultured under similar conditions. An example is shown in Fig. 1, and a summary of analyses of 10 HAM patients (HAM1–10) and 3 normal individuals is shown in Table I.

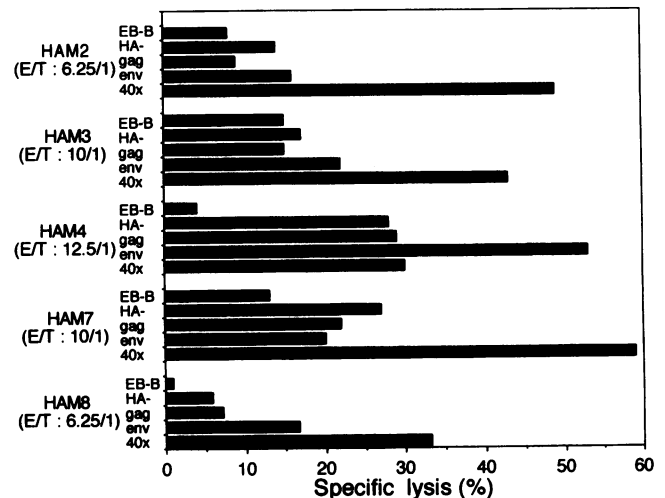


Figure 6. Cytolytic activity of lymphocytes from HAM patients responding to HTLV-I genome products. PBMC from five HAM patients (HAM2, 3, 4, 7, and 8) cultured for 2 wk were examined for the specific lysis against <sup>51</sup>Cr-labeled autologous EB-B cells infected with HTLV-I vaccinia recombinants, LO5-HA<sup>-</sup>, LO5-gag, LO5-env, or LO5-40x as described in Methods (designated as HA-, gag, env, and 40x, respectively, in figure). Uninfected cells (EB-B) were also included as target cells. Data are presented as percentage of specific lysis at E:T ratio ranging from 6.25 to 12.5.

The TCR V $\alpha$  and V $\beta$  genes of these CD8<sup>+</sup> T cells were examined to see if dominantly growing CD8<sup>+</sup> T cells derived from HAM patients are composed of limited clones. The RNA was prepared from lymphocytes cultured for 2 wk, and RT-PCR was performed to amplify the TCR V $\alpha$  and V $\beta$  gene families using the primers listed in Fig. 2 (28, 29). After 22 PCR cycles, Southern blots were performed with the oligo probes listed in Fig. 2. The radioactive intensity of the bands in the Southern blots was also measured using the Bio-Imaging Analyzer (BAS 2000; Fuji Photo Film Co.) for a more quantitative evaluation. A very limited number of TCR V gene families were detected in samples derived from HAM patients, although in cultured normal lymphocytes all or most TCR V gene families were constantly detectable. Examples of Southern blots are shown in Figs. 3 and 4. Similar results were also obtained in all 10 HAM patients examined for both V $\beta$  and V $\alpha$  genes.

Repeated experiments with samples taken on different occasions from the same patients showed essentially similar results (Fig. 5). This indicates that the dominance of a limited number of V gene families in cultured lymphocytes is predetermined in each patient rather than due to artificial variations among culture conditions.

**Presence of CTL against HTLV-I genome products in short-term cultured PBMC derived from HAM patients.** We examined whether cultured lymphocytes of HAM patients were reactive with HTLV-I genome products. Autologous EB-B cell lines derived from HAM patients were infected with recombinant vaccinia viruses to selectively express HTLV-I *gag*, *env*, and *pX* (p40<sup>int</sup>) genes. As shown in Fig. 6, lymphocytes derived from four HAM patients (HAM2, 3, 7, and 8) were specifically cytotoxic against p40<sup>int</sup>-expressing cells and those derived from HAM4 were cytotoxic against env-expressing cells.

**Dominant CD8<sup>+</sup> T cells are CTL for HTLV-I genome products.** In patients HAM2 and HAM8, V $\beta$ 8 and/or V $\beta$ 12 were

Table III. Nucleotide Sequences of TCRs Using V $\beta$ 8.1 and V $\beta$ 12.1 in Uncultured PBMC from HAM2 and HAM8

TCR V $\beta$	Number of clones	Sequences			
		V	N D N	J	C
<b>HAM 2</b>					
V $\beta$ 8.1	10/15*	V $\beta$ 8.1...TGTGCCAGCATCTAGGATGGGGGG		J $\beta$ 2.7	C $\beta$ 2
	2/15	V $\beta$ 8.1...TGTGCCAGCAGTTTGGGTTGGGG		J $\beta$ 2.3	C $\beta$ 2
	1/15	V $\beta$ 8.1...TGTGCCAGAATAACAAGGACAGGGAA		J $\beta$ 1.1	C $\beta$ 1
	1/15	V $\beta$ 8.1...TGTGCCAGGATAACAAGGACAGGGAA		J $\beta$ 1.1	C $\beta$ 1
	1/15	V $\beta$ 8.1...TGTGCCAGCAGTTTAGGATGGCC		J $\beta$ 2.3	C $\beta$ 2
V $\beta$ 12.1	13/15	V $\beta$ 12.1...TGTGCCATCAGTACTCAGGGGGCAT		J $\beta$ 1.1	C $\beta$ 1
	1/15	V $\beta$ 12.1...TCCGCCAGCAGTCGCCTTTGGGGCAGGA		J $\beta$ 1.6	C $\beta$ 1
	1/15	V $\beta$ 12.1...TCCGCCAGCAGTGGAGTCGACGG		J $\beta$ 2.7	C $\beta$ 2
<b>HAM 8</b>					
V $\beta$ 8.1	1/10	V $\beta$ 8.1...TGTGCCAGCAGTTGGGACATC		J $\beta$ 1.3	C $\beta$ 1
	1/10	V $\beta$ 8.1...TGTGCCAGCAGCTTCTGGGGATAC		J $\beta$ 2.2	C $\beta$ 2
	1/10	V $\beta$ 8.1...TGTGCCAGAAGTTCGGCAGGGGGCATT		J $\beta$ 2.7	C $\beta$ 2
	1/10	V $\beta$ 8.1...TGTGCCAGGAGTTTATTACAGCCCCC		J $\beta$ 2.7	C $\beta$ 2
	1/10	V $\beta$ 8.1...TGTGCCAGCATGGCCGGACTGTAAAC		J $\beta$ 2.1	C $\beta$ 2
	1/10	V $\beta$ 8.1...TGTGCCAGCAGTTCACGGACAGGTTTG		J $\beta$ 1.2	C $\beta$ 1
	1/10	V $\beta$ 8.1...TGTGCCAGCAGTCTCGGGTGGGGAGAT		J $\beta$ 2.1	C $\beta$ 2
	1/10	V $\beta$ 8.1...TGTGCCAGAAGTCTCGGGTGGGGAGAT		J $\beta$ 2.1	C $\beta$ 2
	1/10	V $\beta$ 8.1...TGTGCCAGGAGTCCAGGACACTCCGGTGGAGGC		J $\beta$ 1.1	C $\beta$ 1
	1/10	V $\beta$ 8.1...TGTACCAGCAGTATCAGAAGGGGAGAG		J $\beta$ 1.5	C $\beta$ 1

Total RNA was prepared from uncultured PBMC from HAM2 and HAM8. TCRs using V $\beta$ 8.1 and V $\beta$ 12.1 were amplified from cDNA by PCR with V $\beta$ 8.1 or V $\beta$ 12.1 and C $\beta$  primers. Multiple isolates from each cDNA were randomly selected and sequenced. The sequences of V $\beta$ 8.1, V $\beta$ 12.1, J $\beta$ , and C $\beta$  agreed with those reported previously (30, 31). \* Number of clones with sequences shown in the table/number of clones examined.

dominant according to RT-PCR (V $\beta$ 8 and V $\beta$ 12 in HAM2 and V $\beta$ 8 in HAM8). Since mAbs for TCR V $\beta$ 8 and TCR V $\beta$ 12 were available, we subsequently analyzed these patients in detail. Fig. 7 shows that V $\beta$ 8<sup>+</sup> and/or V $\beta$ 12<sup>+</sup> T cells were dominant according to flow cytometry analysis with these mAbs, which agreed with the results of the RT-PCR. The relevance of V $\beta$ 8<sup>+</sup> and/or V $\beta$ 12<sup>+</sup> T cells to p40<sup>tax</sup>-specific CTL was then examined. Lymphocytes treated with mAb for V $\beta$ 8, V $\beta$ 12, CD4, and CD8 were eliminated by panning, and the residual CTL activities against p40<sup>tax</sup>-expressing cells were examined. As shown in Fig. 8, cells of HAM2 pretreated with the mAbs for V $\beta$ 8 or V $\beta$ 12 had significantly reduced CTL activities, and those pretreated with a combination of anti-V $\beta$ 8 and anti-V $\beta$ 12 mAbs, or anti-CD8 mAb, virtually lost all CTL activities. In the similar experiment with HAM8 lymphocytes, cells pretreated either with anti-V $\beta$ 8 mAb or with anti-CD8 mAb virtually lost CTL activity for p40<sup>tax</sup>-expressing cells. These results indicate that V $\beta$ 8<sup>+</sup> CD8<sup>+</sup> and V $\beta$ 12<sup>+</sup> CD8<sup>+</sup> T cells in HAM2 and V $\beta$ 8<sup>+</sup> CD8<sup>+</sup> T cells in HAM8 are CTL for p40<sup>tax</sup>-expressing cells.

*Clonality of CD8<sup>+</sup> CTL for HTLV-I genome products.* The clonality of V $\beta$ 8<sup>+</sup> and/or V $\beta$ 12<sup>+</sup> cells in cultured HAM2 or HAM8 lymphocytes was more precisely analyzed by sequencing V $\beta$ 8<sup>+</sup> cDNA and V $\beta$ 12<sup>+</sup> cDNA. In HAM2, in both V $\beta$ 8<sup>+</sup> cDNA and V $\beta$ 12<sup>+</sup> cDNA, 10 out of 10 randomly selected cDNA clones had identical sequences in each group. In HAM8,

11 out of 12 V $\beta$ 8<sup>+</sup> cDNA clones shared very similar sequences. 5 out of 12 clones shared an identical sequence and another 4 clones displayed another sequence. These two sequences are different only in one amino acid. The remaining two cDNA clones are different from these sequences only in two amino acids (shown in Table II). Taken together, these results indicate oligoclonal characteristics of dominant CD8<sup>+</sup> T cells.

*Presence of clonal expansion of CD8<sup>+</sup> CTL clones in uncultured PBMC of HAM2.* We followed the kinetics of the proportion of V $\beta$ 8<sup>+</sup> or V $\beta$ 12<sup>+</sup> cells after initiating cultured lymphocytes (Fig. 9). The numbers of both V $\beta$ 8<sup>+</sup> CD8<sup>+</sup> and V $\beta$ 12<sup>+</sup> CD8<sup>+</sup> cells were much higher than those of normal individuals (ranging from 0.5 to 3.5%) in uncultured PBMC derived from HAM2. V $\beta$ 8<sup>+</sup> cells accounted for 10% of the total CD8<sup>+</sup> T cells, and the proportion of V $\beta$ 12<sup>+</sup> cells reached 21% of total CD8<sup>+</sup> cells. The ratio increased further with the associated increase of IL-2R $\alpha$  expression, but not IL-2R $\beta$ , during in vitro incubation of HAM2 PBMC (data not shown). In HAM8, V $\beta$ 8<sup>+</sup> CD8<sup>+</sup> T cells accounted for 3% in uncultured PBMC with no significant increase. Randomly selected V $\beta$ 8<sup>+</sup> or V $\beta$ 12<sup>+</sup> cDNA clones derived from uncultured PBMC of HAM2 and HAM8 were sequenced. In HAM2, the sequences of 10 out of 15 V $\beta$ 8<sup>+</sup> cDNA and 13 out of 15 V $\beta$ 12<sup>+</sup> cDNA were identical with those of the corresponding cDNA determined in cultured lymphocytes (Tables II and III). This indicates that about 7 and 18% of the

Table IV. Dominant TCR V Genes in Cultured PBMC of HAM Patients and Their HLA Types

HAM patient	HLA	Dominant TCR V families	
		V $\beta$	V $\alpha$
1	A2 A29 B51 BW54 CW1 DR4 DR9	2, 10	2, 3
2	A24 A26 B51 BW61 CW3 DRW6 DR9	7, 8, 12	15
3	A24 A31 B51 BW60 CW3 DR1 DRW6	3, 7	15
4	A24 B7 BW60 CW3 CW7 DR1 DRW6	3, 4, 6	3, 17
5	A26 A30 B51 DR1 DR4	4, 6, 9, 20	3, 12
6	A24 A31 B51 B7 CW7 DR1 DRW12	18	1, 2
7	A24 AW33 B44 BW54 CW1 DRW6 DRW8	16, 17	2, 3
8	A30 AW33 B44 B51 DR1 DRW6	3, 8, 17	6, 7
10	A2 A24(9) BW54(W22) BW60(40) CW1 CW3 DR4 DRW11(5)	1, 6, 14	12, 14

PBMC from nine HAM patients were cultured for 2 wk in RPMI 1640 with 20% FCS and 400 IU/ml of rIL-2, and the total RNA and cDNA were prepared. 22 PCR cycles were performed using TCR V $\alpha$  5'-primers and the C $\alpha$  3'-primer or TCR V $\beta$  5'-primers and the C $\beta$  3'-primer, then PCR products were separated on an acrylamide gel and stained with ethidium bromide. The expression of TCR V families was considered dominant when a PCR band was clearly visualized after ethidium bromide staining.

total CD8<sup>+</sup> T cells are CTL clones and their precursors with V $\beta$ 8 and V $\beta$ 12, respectively. On the other hand, all of 10 randomly picked up V $\beta$ 8<sup>+</sup> cDNA in HAM8 were mutually different; only one clone was identical with the five clones found in cultured lymphocytes as described before (Tables II and III).

*Absence of common dominant V gene families in cultured HAM lymphocytes.* Since dominant V gene families in cultured lymphocytes derived from HAM patients likely represent those of TCR on CTL against HTLV-I genome products, the usage of TCR genes in 10 patients was examined to determine whether there is any common V gene usage. The dominance of TCR V gene usage in these patients was also analyzed with respect to the patients' HLA haplotypes. No particular tendency was observed in either analysis (Table IV).

## Discussion

The presence of CD8<sup>+</sup> CTL for HTLV-I<sup>+</sup> cells in PBMC derived from HAM has been repeatedly reported (32–35). These CTL are often directed to p40<sup>tax</sup> though CTL, for other genome products such as *env* and *pol* have also been detected. Similar CTL have been found, though with somewhat weaker activity, in PBMC derived from asymptomatic HTLV-I carriers. The short-term cultivation of these PBMC with no exogenous antigen stimulation often results in enhanced CTL activity. This evidence indicates that the immune system in HTLV-I–infected

hosts is constantly exposed to stimulation by HTLV-I genome products.

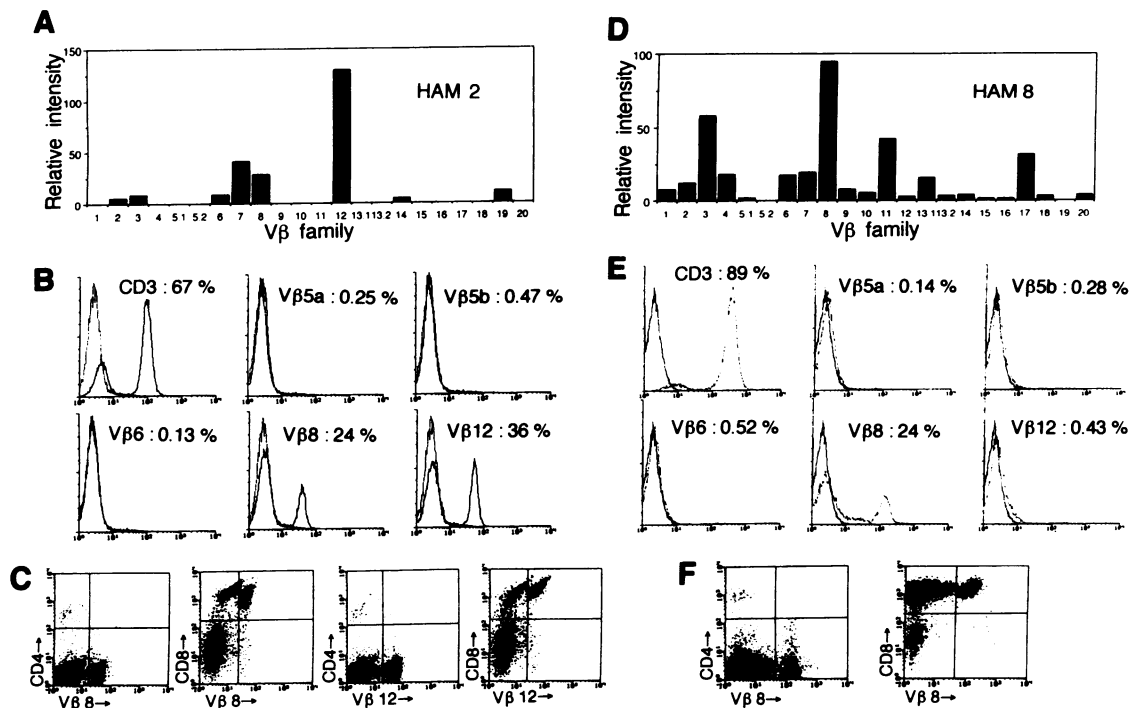
Along with the above reported evidences, results of this study indicate that CD8<sup>+</sup> T cells reactive for HTLV-I genome products become dominant during a short-term in vitro cultivation. CD4<sup>+</sup> T cells infected with HTLV-I probably express HTLV-I genomes during cultivation, and CD8<sup>+</sup> T cells presensitized in vivo with their genome products continuously expand in culture as a consequence of exposure to the cognate antigens. HTLV-I–infected CD8<sup>+</sup> T cells are known to express viral genome products including p40<sup>tax</sup> shortly after initiation of culture. Infected CD4<sup>+</sup> T cells are therefore activated via a potent *trans*-activation of p40<sup>tax</sup> (36–38). These infected CD4<sup>+</sup> T cells may function as potent antigen-presenting cells by presenting both target peptides derived from HTLV-I genome products and accessory signals including various cytokines whose production was promoted by the *trans*-activation of p40<sup>tax</sup>. Cellular immune reactions for viral peptides in HTLV-I–infected individuals might be therefore unique among various viruses.

The striking finding was that the peripheral blood of HAM2 contained an extraordinarily high proportion of two CTL clones and their precursors for p40<sup>tax</sup>. V $\beta$ 8<sup>+</sup> CD8<sup>+</sup> T and V $\beta$ 12<sup>+</sup> CD8<sup>+</sup> T cells constituted 10 and 21% of the total CD8<sup>+</sup> T cells, respectively, and ~ 67% of V $\beta$ 8<sup>+</sup> (10 out of 15 cDNA clones) and 87% of V $\beta$ 12<sup>+</sup> cells (13 out of 15 cDNA clones) were p40<sup>tax</sup> specific CTL clones and their precursors. HAM2 constantly presents white blood cell counts in peripheral blood ranging from 3,600/cmm to 3,900/cmm, with lymphocyte counts ranging from 1,500/cmm to 2,000/cmm, and therefore the relative increase of these two clones was not due to lymphopenia. The absence of integration of viral genomes in short-term cultured CD8<sup>+</sup> T cells of HAM2 (data not shown) denies the possible clonal expansion of two clones as a consequence of transformation by HTLV-I.

A somewhat similar finding has been reported by MacDonald et al. (39). DBA/2 mice developed CTL clones reactive with HLA-CW3 transfected into syngeneic P815 tumor cells, which revealed a striking conservation of the TCR structure with V $\beta$ 10 and J $\alpha$  pHDS58 segments. In a primary response to HLA-CW3–transfected P815, a dramatic expansion of V $\beta$ 10<sup>+</sup> CD8<sup>+</sup> CTL occurred in various lymphoid organs, reaching 65% in peripheral blood lymphocytes. These CTL presented restricted V $\alpha$  domains, a dominant J $\alpha$  segment, and a conserved CDR3 length for both  $\alpha$  and  $\beta$  chains in a majority of responding cells. Our results, along with these findings in mice, indicate that T cell immune responses to certain antigens may reach a range far beyond expectation as a consequence of the extremely high efficiency of clonal expansion.

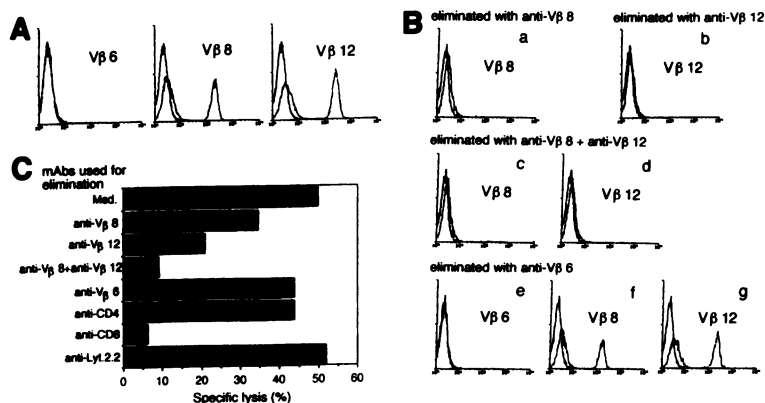
A relatively high viral load of HAM patients, when compared with asymptomatic healthy carriers, has been reported by Yoshida et al. (40) and by us (41). It has been also pointed out that HAM patients may be classified as high responders in terms of immune responses for HTLV-I as well as other antigens. Whether a high immune response for HTLV-I in HAM patients is relevant to disease pathogenesis remains an open question. Since there is little evidence on viral integration in the nervous system, the possibility that HTLV-I–specific CTL directly attack the spinal cord might be limited unless cross-reactive peptides are expressed. The elimination of cells infected with viruses by means of T cell immunity is conceivable for a variety of viruses. Eradicating cancer cells by enhancing T cell immunity is also becoming more realistic since genes coding peptides



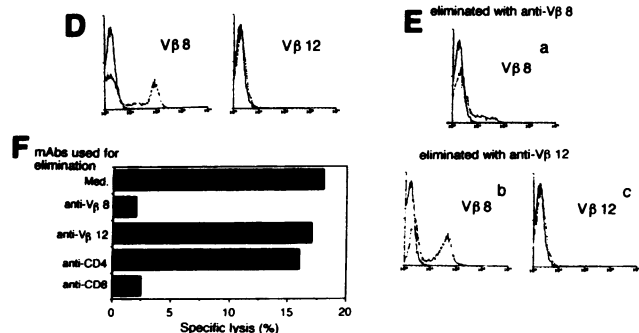


**Figure 7.** The surface expression of the TCR V $\beta$  family examined by flow cytometry. PBMC derived from HAM2 and HAM8 were cultured for 2 wk and then were analyzed. (A and D) 22 cycles of PCR for the V $\beta$  family were performed followed by Southern blotting. The relative intensity of RT-PCR products is shown as described in Fig. 4. (B and E) Cultured PBMC from HAM2 (B) and HAM8 (E) were stained with the indicated mAbs and then with FITC-conjugated goat anti-mouse IgG. The results were expressed as fluorescence intensity versus the number of cells. (C and F). Two-color fluorescence analysis of cultured PBMC from HAM2 (C) and HAM8 (F). Anti-TCR V $\beta$ 8-FITC or anti-TCR V $\beta$ 12-FITC and anti-CD4-PE or anti-CD8-PE were used as indicated.

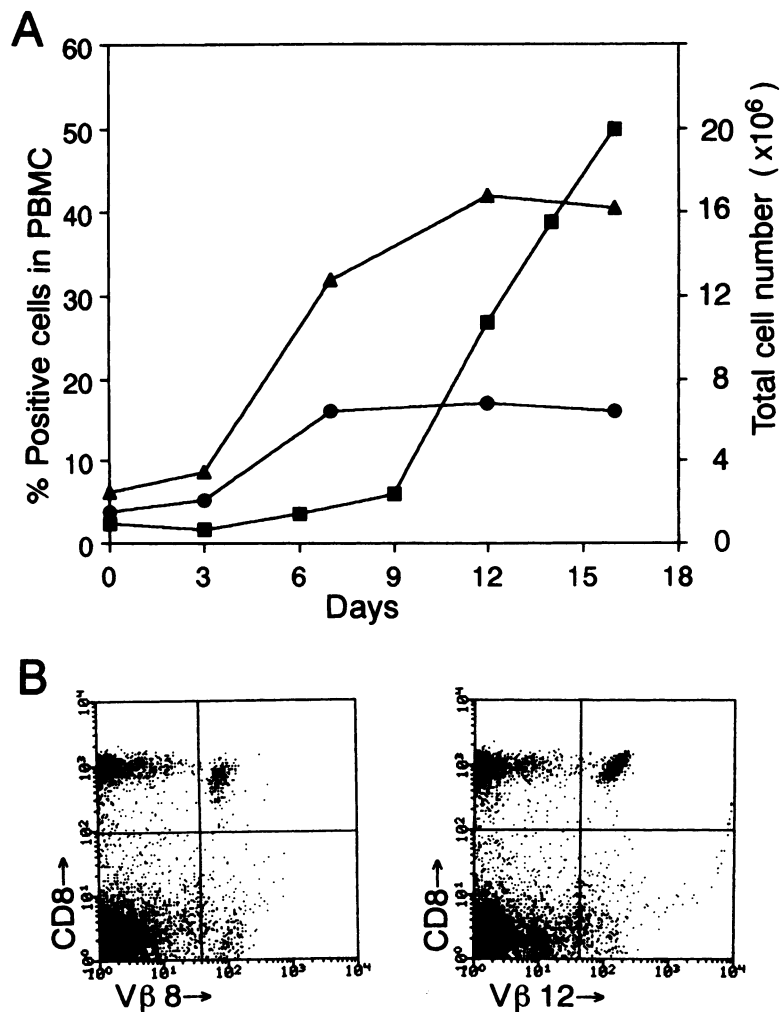
## HAM 2



## HAM 8



**Figure 8.** The cytolytic activity of cultured lymphocytes from HAM2 and HAM8 is dependent on V $\beta$ 8<sup>+</sup> CD8<sup>+</sup> or V $\beta$ 12<sup>+</sup> CD8<sup>+</sup> cells. (A and D) Surface expression of TCR V $\beta$ 6, V $\beta$ 8, V $\beta$ 12 on cultured PBMC from HAM2 (A) and HAM8 (D). PBMC were cultured for 2 wk and stained with anti-TCR V $\beta$ 6-FITC, anti-TCR V $\beta$ 8-FITC, or anti-TCR V $\beta$ 12-FITC mAbs. (B) Cultured PBMC from HAM2 were eliminated by panning using anti-TCR V $\beta$ 8 (a), anti-TCR V $\beta$ 12 (b), anti-TCR V $\beta$ 8 and anti-TCR V $\beta$ 12 (c and d), or anti-TCR V $\beta$ 6 (e-g) as a negative control and then stained with anti-TCR V $\beta$ -FITC mAbs as indicated in each histogram, to confirm the elimination of cells with each phenotype. The results were expressed as fluorescence intensity versus the number of cells. (C) After elimination, remaining CTL activity against autologous EB-B cells infected with the HTLV-I vaccinia recombinant, LO5-40x, was examined. Data are presented as percentage of specific lysis at E:T ratio (3.75:1). (E) Cultured PBMC from HAM8 were eliminated by panning using anti-TCR V $\beta$ 8 (a) or anti-TCR V $\beta$ 12 (b and c) as a negative control and then stained with indicated mAbs. (F) After elimination, remaining CTL activity against HTLV-I p40<sup>max</sup> was examined (E:T ratio, 10:1).



**Figure 9.** The kinetics of cell growth and the percentage of Vβ8<sup>+</sup> or Vβ12<sup>+</sup> cells in vitro cultured PBMC derived from HAM2. (A) PBMC were cultured in vitro initially at a density of 10<sup>6</sup>/ml. The number of total cells (■) in culture was counted. The percentages of Vβ8<sup>+</sup> cells (●) and Vβ12<sup>+</sup> cells (▲) in the cultured cells were measured by using a FACS<sup>®</sup> and anti-Vβ8-FITC and anti-Vβ12-FITC. (B) Two-color fluorescence analysis of uncultured PBMC from HAM2. Anti-TCR Vβ8-FITC or anti-TCR Vβ12-FITC and anti-CD8-PE were used as indicated.

for human melanoma-specific CTL have been defined (42, 43). The manipulation of immune systems with appropriate antigens and other signals may generate the clonal expansion of such CTL to the magnitude reported here and may eventually eliminate virally infected as well as cancer cells.

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