[13] N. Maeda, Y. Takeuchi, M. Takada, Y. Namba, N. Oku, Synthesis of angiogenesis-targeted peptide and hydrophobized polyethylene glycol conjugate, Bioorg. Med. Chem. Lett. 14 (4) (2004) 1015–1017.

[14] N. Maeda, Y. Takeuchi, M. Takada, Y. Sadzuka, Y. Namba, N. Oku, Anti-neovascular therapy by use of tumor neovasculature-targeted long-circulating liposome, J. Control. Release 100 (1) (2004) 41–52.

[15] N. Maeda, S. Miyazawa, K. Shimizu, T. Asai, S. Yonezawa, S. Kitazawa, Y. Namba, H. Tsukada, N. Oku, Enhancement of anticancer activity in antineovascular therapy is based on the intratumoral distribution of the active targeting carrier for anticancer drugs, Biol. Pharm. Bull. 29 (9) (2006) 1936–1940.

[16] C.H. Crane, L.M. Ellis, J.L. Abbruzzese, C. Amos, H.Q. Xiong, L. Ho, D.B. Evans, E.P. Tamm, C. Ng, P.W. Pisters, C. Charnsangavej, M.E. Delclos, M. O'Reilly, J.E. Lee, R.A. Wolff, Phase I trial evaluating the safety of bevacizumab with concurrent radiotherapy and capecitabine in locally advanced pancreatic cancer, J. Clin. Oncol. 24 (7) (2006) 1145–1151.

[17] H.L. Kindler, G. Friberg, D.A. Singh, G. Locker, S. Nattam, M. Kozloff, D.A. Taber, T. Karrison, A. Dachman, W.M. Stadler, E.E. Vokes, Phase II trial of bevacizumab plus gemcitabine in patients with advanced pancreatic cancer, J. Clin. Oncol. 23 (31) (2005) 8033–8040.

[18] N. Oku, Anticancer therapy using glucuronate modified long-circulating liposomes, Adv. Drug Deliv. Rev. 40 (1–2) (1999) 63–73.

[19] D.T. Auguste, R.K. Prud'homme, P.L. Ahl, P. Meers, J. Kohn, Association of hydrophobically-modified poly(ethylene glycol) with fusogenic liposomes, Biochim. Biophys. Acta 1616 (2) (2003) 184–195.

[20] H. Maeda, J. Wu, T. Sawa, Y. Matsumura, K. Hori, Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review, J. Control. Release 65 (1–2) (2000) 271–284.

[21] R.J. Giordano, M. Cardo-Vila, J. Lahdenranta, R. Pasqualini, W. Arap, Biopanning and rapid analysis of selective interactive ligands, Nat. Med. 7 (11) (2001) 1249–1253.

Reduced Frequency, Diversity, and Function of Human T Cell Leukemia Virus Type 1-Specific CD8^{+} T Cell in Adult T Cell Leukemia Patients[1]

Tomohiro Kozako,* Naomichi Arima,[2]* Shingo Toji,[¶] Izumi Masamoto,* Masaki Akimoto,* Heiichiro Hamada,[‡] Xiao-Fang Che,[§] Hiroshi Fujiwara,[‡] Kakushi Matsushita,[‡] Masahito Tokunaga,* Koichi Haraguchi,* Kimiharu Uozumi,* Susumu Suzuki,[†] Toshiro Takezaki,[†] and Shunro Sonoda[†]

Human T cell lymphotropic virus type 1 (HTLV-1)-specific CTL are thought to be immune effectors that reduce the risk of adult T cell leukemia (ATL). However, in vivo conditions of anti-HTLV-1 CTL before and after ATL development have yet to be determined. To characterize anti-HTLV-1 CTL in asymptomatic HTLV-1 carriers (AC) and ATL patients, we analyzed the frequency and diversity of HTLV-1-specific CD8^{+} T cells in PBMC of 35 AC and 32 ATL patients using 16 distinct epitopes of HTLV-1 Tax or Env/HLA tetramers along with intracellular cytolytic effector molecules (IFN-γ, perforin, and granzyme B). Overall frequency of subjects possessing Tax-specific CD8^{+} T cells was significantly lower in ATL than AC (53 vs 90%; $p = 0.001$), whereas the difference in Env-specific CD8^{+} T cells was not statistically significant. AC possessed Tax_{11-19}/HLA-A*0201-specific tetramer^{+} cells by 90% and $Tax_{301-309}$/HLA-A*2402-specific tetramer^{+} cells by 92%. Some AC recognized more than one epitope. In contrast, ATL recognized only Tax_{11-19} with HLA-A*0201 and $Tax_{301-309}$ with HLA-A*2402 at frequencies of 30 and 55%. There were also significant differences in percentage of cells binding Tax_{11-19}/HLA-A*0201 and $Tax_{301-309}$/HLA-A*2402 tetramers between AC and ATL. Anti-HTLV-1 Tax CD8^{+} T cells in AC and ATL produced IFN-γ in response to Tax. In contrast, perforin and granzyme B expression in anti-HTLV-1 CD8^{+} T cells of ATL was significant lower than that of AC. Frequency of Tax-specific CD8^{+} T cells in AC was related to proviral load in HLA-A*0201. These results suggest that decreased frequency, diversity, and function of anti-HTLV-1 Tax CD8^{+} T cell clones may be one of the risks of ATL development. *The Journal of Immunology*, 2006, 177: 5718–5726.

A
dult T cell leukemia (ATL)[3] is caused by infection with human T cell lymphotropic virus type 1 (HTLV-1) (1–3), a retrovirus infecting ~10–15 million people world-wide, in southern Japan, the Caribbean basin, South America, Melanesia, and equatorial Africa (4). More than 800 cases of ATL are diagnosed each year in Japan (5). Although there has been recent progress in chemotherapy for ATL, with the LSG15 protocol showing an overall 5-year survival rate of 17.5% (6), the prognosis of ATL is still poor. The recent report of successful, sustained complete remission of ATL by hemopoietic stem cell transplantation led to the hypothesis that immunocompetency reconstructed by stem cell transplantation may regenerate cytotoxic immune effectors against HTLV-1-tranformed T cell and related tumor Ags, and then induce a graft-vs-leukemia reaction in ATL patients (7, 8).

HTLV-1-specific CTL plays an important role in suppressing proliferation of HTLV-1-infected or -transformed T cells in vitro (9–12) and thus may prevent development of ATL (13, 14). Because ATL develops in ~2% of people infected with HTLV-1 after a long latent period (15), it is possible that CTL fails in only a fraction of HTLV-1 carriers with a specific immunogenetic background (16–18). Previously, we identified HTLV-1 Tax epitopes recognized by HLA class I molecules using PMBC of asymptomatic HTLV-1 carriers (AC) in vitro and reported that the frequencies of HLA alleles lacking epitope anchor motifs, HLA-A*26, HLA-B*4002, HL-B*4006, and HLA-B*4801, were higher in ATL patients than in AC. These findings suggested that insufficient generation of CTL allowed outgrowth of HTLV-1-transformed cells in the host (19). Indeed, ATL patients produced anti-Tax CD8^{+} T cell in short-term cultivation, although their IFN-γ production was insufficient (14). These findings suggested a key role of anti-HTLV-1 Tax CTL in prevention of ATL leukemogenesis.

The chromium-51 (^{51}Cr) release assay and the calcein acetoxymethyl fluorescence assay are the most widely used methods for estimating CD8^{+} CTL (19–23). However, these assays are not suitable for mass screening of clinical samples because they are time-consuming. In addition, these assays do not reflect the in vivo status because they require short-term culture. Infected or leukemic cells easily produce HTLV-1 Tax protein as rapidly as within

*Division of Host Response, Center for Chronic Viral Diseases, Graduate School of Medical and Dental Sciences, Kagoshima University; [†]International Island and Community Medicine, Graduate School of Medical and Dental Sciences, Kagoshima University; [‡]Department of Hematology and Immunology, Kagoshima University Hospital, and [§]Department of Molecular Oncology, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan; and [¶]Medical and Biological Laboratories, Co., Ltd, Nagoya, Japan

[3] Abbreviations used in this paper: ATL, adult T cell leukemia; HTLV-1, human T cell lymphotropic virus type 1; AC, asymptomatic HTLV-1 carrier; 7-AAD, 7-aminoactinomycin D.

a few hours in culture (12, 24–26). Flow cytometric assay with HLA tetramers in combination with intracellular IFN-γ, perforin, and granzyme B, which play important and diverse roles in controlling many viral infections (27, 28), is useful because of its simplicity, specificity, and sensitivity in detection of Ag-specific CTL in PMBC in vivo or in vitro (29–31).

In the present study, we developed 14 HTLV-1/HLA tetramers to detect anti-HTLV-1 CD8$^+$ CTL clones. To characterize the in vivo status of anti-HTLV-1 CD8$^+$ CTL in AC and ATL patients, we analyzed clonal frequency, diversity, and function of anti-HTLV-1 CD8$^+$ CTL in freshly isolated PBMC by examining the 16 distinct HTLV-1/HLA tetramers with HLA-A*0201 and HLA-A*2402 in combination with functional CTL assay for intracellular molecules relevant to cytolytic effector function. We found that frequency, diversity, and function of HTLV-1-specific CD8$^+$ T cell are significantly reduced in ATL.

Materials and Methods

Subjects

The study subjects included 35 AC (22–82 years old; mean, 60.0; male: female, 15:20) and 32 ATL (27–85 years old; mean, 61.2; male:female, 14:18), all of whom were recruited from Kagoshima University Hospital located in southern Kyushu, Japan. All subjects came to the hospital for examination of HTLV-1 infection and clinical checkup. They were examined by standard serological testing for HTLV-1 and hematological examinations for ATL. Those patients positive for HTLV-1 serology but with no clinical symptoms of ATL (32) or other HTLV-1-related diseases (33) were designated as AC. Diagnosis of ATL was made according to Shimoyama's criteria (32). We tested PBMC of all ATL patients before treatment. All subjects were inhabitants of Kagoshima prefecture, southern Kyushu, Japan, where ATL is endemic. All subjects signed an informed consent to participate in the present study and to allow review of their medical records, and gave a sample of peripheral blood for HLA typing and HLA tetramer assay. The study protocol was reviewed and approved by the Medical Ethical Committee of Kagoshima University. By HLA typing, 88% of the HTLV-1-infected subjects (AC and ATL combined) were shown to have HLA-A*02 or HLA-A*24. HLA allele types were representative of the population of Southern Kyushu (34). Of the 35 AC, 10 had HLA-A*0201, 24 had HLA-A*2402, 3 had both alleles in heterozygous combination, and 4 included as negative controls had neither HLA-A*0201 nor HLA-A*2402. Of the 32 ATL patients, 10 had HLA-A*0201, 22 had HLA-A*2402, 4 had both alleles in heterozygous combination, and 4 included as negative controls had neither HLA-A*0201 nor HLA-A*2402.

Preparation of PBMC

PBMC were obtained from peripheral blood by separating on Ficoll Hypaque (Amersham Biosciences) density gradient centrifugation at 400 × g for 30 min, followed by washing three times with 1% FCS RPMI 1640 at 200 × g centrifugation for 10 min to remove residual platelets. The fresh PBMC were used for tetramer assay and in vitro expansion of anti-HTLV-1 CD8$^+$ CTL. The remaining PBMC were cryopreserved in liquid nitrogen until examination as described previously (35).

HLA typing of PBMC

Subjects positive for HLA-A*02 and A*24 were screened by serological staining with mAbs for HLA-A*02 supertype (clone BB7.2) and HLA-A*24 supertype (clone 17A10) (Medical and Biological Laboratories), followed by secondary staining with goat anti-mouse IgG-FITC (Immunotech) according to the manufacturer's instruction and subjected to flow cytometry on a FACScan (BD Biosciences). HLA allele types were determined by the PCR-sequence-specific oligonucleotide probes protocols with the Luminex 100 xMAP flow cytometry dual-laser system method using high m.w. DNA isolated from the cryopreserved PBMC as described (G&G Science) (36). Briefly, amplification was as follows: target DNA was PCR-amplified using 5'-biotin-labeled primers that are highly specific to certain sequences of HLA genes. Hybridization was as follows: after denaturation at 95°C, amplified DNA was allowed to hybridize to complementary DNA probes coupled to microbeads. Streptavidin-PE reaction was as follows: the hybridized PCR product on the oligobeads was labeled with streptavidin-PE. Measurement was as follows: Luminex apparatus identified the fluorescent intensity of PE on each coded oligobead that has hybridized with the biotin-labeled PCR product. Genosearch typing soft-

ware (G&G Science) assisted in determining the HLA genotype (alleles) of the sample DNA.

Preparation of HTLV-1 Tax, Env/HLA tetramers

A total of 16 distinct HTLV-1/HLA tetramers were used in the present study (Table I). We originally developed 14 distinct PE-conjugated HLA-A*0201 and HLA-A*2402 tetramers for four Tax and three Env peptides. These Tax and Env peptides were selected based on our own published data of CTL epitope mapping for HTLV-1 Tax and Env peptides in vitro (19). Two additional HTLV-1/HLA tetramers for Tax peptides were purchased from Beckman Coulter. We and Beckman Coulter developed tetramers by the same procedure. HLA tetramers were produced as described previously (37, 38). Briefly, recombinant β_2-microglobulin and the extracellular portion of the HLA class I H chain containing the BirA recognition sequence in frame at its C terminus were expressed in Escherichia coli as insoluble aggregates that formed inclusion bodies. Purified inclusion bodies were solubilized in urea, and monomeric peptide-HLA class I complexes were refolded around peptides by dilution denaturing conditions. After buffer exchange, a specific lysine residue in the H chain C-terminal tag was biotinylated with the BirA enzyme. Monomeric complexes were purified by monomeric avidin gel chromatography (Pierce). Tetrameric arrays of biotinylated peptide-HLA class I complexes were formed by the addition of PE-labeled streptavidin (Prozyme) at a molar ratio of 4:1. The purity of each 16 HLA tetramer was tested by HPLC and its binding affinity was evaluated from the BIMAS score at Medical and Biological Laboratories.

Detection of anti-HTLV-1 CD8$^+$ T cells in PBMC using tetramer

The procedure was slightly changed from Haanen et al. (39) and Skinner et al. (40). We used Alexa Fluor 488-labeled anti-CD8 mAbs and Alexa Fluor 647-labeled mouse-anti-PE mAbs following PE-labeled HTLV-1/HLA tetramer because these dyes provide resistance to photobleaching. Briefly, the PBMC were incubated with PE-labeled HTLV-1/HLA tetramer (20 µg/ml) with normal goat serum for 50 min at 4°C in the dark. The PBMC were washed with PBS, and then incubated with Alexa Fluor 488-labeled anti-CD8 mAbs (6 µg/ml; Caltag Laboratories) and Alexa Fluor 647-labeled mouse-anti-PE mAbs (20 µg/ml; Sigma-Aldrich), which was produced using Alexa Fluor 647 mAbs Labeling kit (Molecular Probes) according to the manufacturer's manuals, for 50 min at 4°C in the dark. Finally, the PBMC were washed three times, and then mounted to slides with 10 µl of ProLong Gold (Molecular Probes). Stained PBMC were visualized using an OYMPAS IX81 confocal microscope.

HTLV-1 Tax, Env/HLA tetramer assay

Aliquots of 1 × 10^6 freshly isolated PBMC or cultured cells were incubated with the HTLV-1/HLA tetramers with each of the 16 distinct HTLV-1 Tax or Env peptides for 45 min at 4°C, followed by staining with FITC-conjugated murine anti-human CD8 mAbs (Beckman Coulter) and anti-CD45-PerCP (BD Biosciences) for 45 min at 4°C according to the manufacturer's instructions (Medical and Biological Laboratories). Aliquots of 1 × 10^5 fresh CD45-positive lymphocytes were performed using

Table I. HTLV-1 Tax, Env/HLA tetramers[a]

Tetramers	HLA Allele	HTLV-1 Peptide	Epitopes
T11	A*0201	Tax$_{11-19}$	LLFGYPVYV
T123	A*0201	Tax$_{123-131}$	TLGQHLPTL
T155	A*0201	Tax$_{155-163}$	YLYQLSPPI
T178	A*0201	Tax$_{178-186}$	QLGAFLTNV
T307	A*0201	Tax$_{307-315}$	LLFEEYTNI
E175	A*0201	Env$_{175-183}$	FLNTEPSQL
E239	A*0201	Env$_{239-247}$	VLYSPNVSV
E442	A*0201	Env$_{442-450}$	ALQTGITLV
T12	A*2402	Tax$_{12-20}$	LFGYPVYVF
T187	A*2402	Tax$_{187-195}$	PYKRIEELL
T289	A*2402	Tax$_{289-297}$	SFLLSHGLI
T301	A*2402	Tax$_{301-309}$	SFHSLHLLF
T311	A*2402	Tax$_{311-319}$	EYTNIPISL
E11	A*2402	Env$_{11-19}$	FFQFCPLIF
E21	A*2402	Env$_{21-29}$	DYSPSCCTL
E153	A*2402	Env$_{153-161}$	HFSKCGFPF

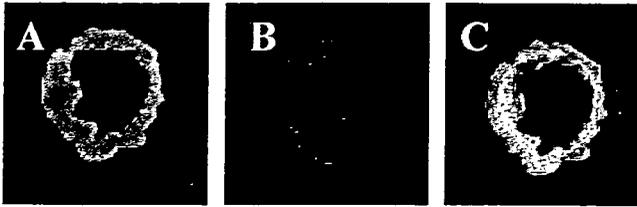[a] HLA binding peptide anchor motifs are shown in bold.

FIGURE 1. Ex vivo detection of anti-HTLV-1 CD8$^+$ T cell. Freshly isolated PBMC were stained with FITC-labeled anti-CD8 mAbs (*A*) and Alexa 647-labeled anti-PE mAbs in combination with PE-labeled HTLV-1/HLA tetramer (*B*). *C*, Colored overlay of *A* and *B*.

FACScan (BD Biosciences) (41) and analyzed with FlowJo software (Tree Star) (28). In the cultured PBMC samples, apoptotic or necrotic cells in the cultures were stained with 7-amino-actinomycin D (7-AAD; Beckman Coulter) (42), and the 7-AAD-positive cells were excluded by FACScan analysis. HIV/HLA tetramer (Medical and Biological Laboratories) for negative and CMV/HLA tetramer (Beckman Coulter) were also stained.

Real-time PCR quantification of HTLV-1 proviral load in PBMC

DNA was extracted from 1 × 10^6 cells of PBMC using SMI TEST EX-R&D (G&G Science). The HTLV-1 proviral load in PBMC was assayed in 64 of 67 subjects by methods of quantitative PCR using a Light Cycler System (Roche Diagnostics) by intraassay using a series of duplicate measurements of 12 test samples with standard DNA of four different dilutions for each assay as described previously (43). In brief, the duplicate intraassay for HTLV-1 provirus load in PBMC was run by simultaneous measurements of β-globin DNA and HTLV-1 DNA using the standard DNA, β-globin DNA from Roche Diagnostics, and HTLV-1 provirus DNA from MT-2 cells. One PBMC has two copies of β-globin gene (equivalent to 6 pg of β-globin DNA) and one MT-2 cell has eight copies of HTLV-1 provirus DNA (equivalent to 6 pg of HTLV-1 in DNA). The β-globin PCR primer and probe sets were commercial kits (Roche Diagnostics). The HTLV-1 primer set corresponded to the highly conserved HTLV-1 pX region, SK43 and SK44. The HTLV-1 pX probe set was designed by ourselves for the two adjacent parts of the pX region, which were labeled with different fluorophores according to the manufacturer's instructions. The HTLV-1 provirus load was expressed as number of copies per 10^3 cells using the following formula: HTLV-1 provirus load = ((HTLV-1 pX copy number)/(β-globin copy number/2)) × 10^3. The detection limit of this method was 0.2 copies of HTLV-1 provirus/10^3 cells.

Induction of HTLV-1 Tax, Env-specific CTL

Aliquots of PBMC (1 × 10^6 cells) were used for in vitro expansion of CD8$^+$ CTL clones in cultures with 2 × 10^{-6} M of distinct HTLV-1 Tax and Env peptides in RPMI 1640 medium supplemented with the following reagents: 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.1 mM nonessential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.05 mM 2-ME, 50 U/ml recombinant human IL-2, and 10% heat-inactivated FCS (RPMI 1640-CM). All culture conditions were the same as described elsewhere (41). The cultured PBMC were examined using the HTLV-1/HLA tetramer assay described above.

Intracellular IFN-γ, perforin, and granzyme B assay

PBMC (1 × 10^6) for IFN-γ analysis were cultured for 16 h with or without 0.02 μM HTLV-1 Tax peptide in combination with brefeldin A (BD Biosciences) in RPMI 1640-CM. Harvested cells for IFN-γ and freshly isolated PBMC for perforin and granzyme B were labeled with HTLV-1/HLA-tetramer-PE and anti-CD8-allophycocyanin Ab (BD Biosciences) for cell surface Ags (44). The cells were further treated with Permeabilizing Solution (BD Biosciences) for 10 min. After washing with buffer containing 0.1% saponin, the cells were incubated with anti-human IFN-γ-FITC, perforin-FITC, or granzyme B-FITC Ab (BD Biosciences) in buffer containing saponin. FastImmune Control γ2aFITC/γ1PE was stained as negative control (BD Biosciences). Aliquots of 1 × 10^4 CD8$^+$ T lymphocytes were performed using FACSCalibur (BD Biosciences) and analyzed with FlowJo software.

CD107a mobilization assay

PBMC (1 × 10^6) were cultured for 6 h with or without 0.02 μM HTLV-1 Tax peptide in combination with anti-CD107a mAbs-FITC (Southern Biotech) and the secretion inhibitor monensin (BD Biosciences) in RPMI 1640-CM (45, 46). Following incubation, cell suspensions were washed with cold PBS. The cells were further stained with tetramer-PE and anti-CD8 mAb-PE-Cy5 (Beckman Coulter) as described above. Aliquots of 1 × 10^4 CD8$^+$ T lymphocytes were performed using FACSCalibur (BD Biosciences) and analyzed with FlowJo software.

Statistical analysis

Differences in HTLV-1-specific CD8$^+$ T cell frequency between AC and ATL patients were evaluated by χ^2 test or exact test. The Mann-Whitney U test was used to compare the percentages of cells binding tetramer$^+$ or tetramer$^+$intracellular cytokine$^+$ in CD8$^+$ lymphocytes between subjects with AC and ATL, between the proviral load of Tax-tetramer positive AC
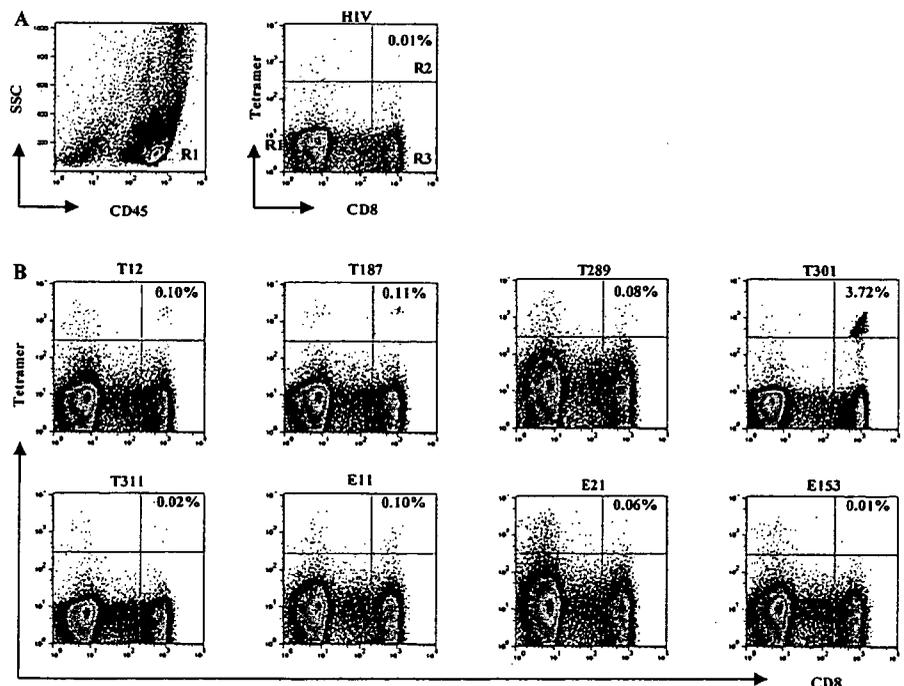

FIGURE 2. Variety of anti-HTLV-1 CD8$^+$ T cells in fresh PBMC. *A*, Tetramer$^+$CD8$^+$ T cells were estimated in CD45$^+$ T lymphocyte region (R1). We observed HIV-tetramer$^+$CD8$^+$ T cells as negative control. *B*, Fresh PBMC isolated from AC were stained with eight distinct HTLV-1/HLA-A*2402-tetramers (T12, T187, T289, T301, T311, E11, E21, and E153). Numbers in the *upper right quadrants* represent the percentages of tetramer$^+$CD8$^+$ T cells in CD8$^+$ CD45$^+$ T lymphocytes (R2/R2+R3).

Table II. *Frequencies of HTLV-1-specific tetramer binding cells in PBMC of AC and ATL patients*

HLA Allele	Tetramers	Positive Epitopes Detected by HTLV-1/HLA Tetramers[a]	
		AC (%)	ATL (%)
A*0201	T11	90 (9/10)[b]	30 (3/10)*
A*0201	T123	0 (0/10)	0 (0/10)
A*0201	T153	0 (0/10)	0 (0/10)
A*0201	T178	10 (1/10)	0 (0/10)
A*0201	T307	10 (1/10)	0 (0/10)
A*0201	E175	0 (0/10)	0 (0/10)
A*0201	E239	0 (0/10)	0 (0/10)
A*0201	E442	0 (0/10)	0 (0/10)
A*2402	T12	17 (4/24)	0 (0/22)
A*2402	T187	13 (3/24)	0 (0/22)
A*2402	T289	0 (0/24)	0 (0/22)
A*2402	T301	92 (22/24)	55 (12/22)*
A*2402	T311	0 (0/24)	0 (0/22)
A*2402	E11	4 (1/24)	0 (0/22)
A*2402	E21	4 (1/24)	0 (0/22)
A*2402	E153	8 (2/24)	0 (0/22)
Tax CTL positives		24 (40/170)	9 (15/160)**
Env CTL positives		4 (4/102)	0 (0/96)
Total CTL positives		16 (44/272)	6 (15/256)**

[a] The percentages of HTLV-1/HLA tetramer^{+}CD8^{+} T cells in the CD8^{+}CD45^{+} T lymphocytes that are ≥0.1% are counted as positives, whereas those 0–0.09% are counted as negatives.

[b] Epitopes detected by HTLV-1/HLA tetramers/number of tetramers tested. Eight tetramers were used for testing in subjects carrying either HLA-A*0201 or HLA-A*2402, whereas those carrying both HLA-A*0201 and HLA-A*2402 were tested with 16 tetramers.

*, $p < 0.01$; **, $p < 0.001$, significant differences between AC and ATL by χ^2 test.

and negative AC. Statistical significance was two-sided at α of 0.05. Values of p were corrected for multiple comparisons using StatView software version 5.0 (SAS Institute).

Results

Specificity and sensitivity of HTLV-1/HLA tetramer assay for anti-HTLV-1 CD8^{+} T cells

The feasibility of the developed HTLV-1/HLA tetramer assay was tested by detection of anti-HTLV-1 CD8^{+} T cells in freshly isolated PBMC from 35 AC and 32 ATL patients. As shown in Figs. 1–4, anti-HTLV-1 CD8^{+} T cells were detected. We could visually detect anti-HTLV-1 CD8^{+} T cells in combination staining with tetramer and CD8 using confocal microscopy (Fig. 1). For specificity test, we observed HIV-tetramer^{+}CD8^{+} cells (R2; Fig. 2A) as negative control in CD8^{+}CD45^{+} T lymphocytes (R2+R3; Fig. 2A) (mean, 0.04; SD, 0.05; 95% confidential interval, 0.06). We also observed 0–0.02% tetramer^{+}CD8^{+} cells in CD8^{+}CD45^{+} T lymphocytes of negative control subjects who had neither HLA-A*0201 nor HLA-A*2402. These HLA tetramers could detect anti-

HTLV-1 CD8^{+} T cells possessing HLA-A*0201 or HLA-A*2402 in circulating PBMC.

Fig. 2 shows representative data of anti-HTLV-1 CD8^{+} T cells in AC possessing HLA-A*2402. Tetramer^{+}CD8^{+} cells were estimated in CD45^{+} T lymphocytes (R1). This subject showed a wide spectrum of HLA tetramer staining with CD8^{+} T cells ranging from 0.01 to 3.72% (R2/R2+R3) in which >0.1% of the positive staining showed a definitely clustered pattern of CD8^{+} T cells (Fig. 2B, T12, T187, T301, and E11), but the subject with <0.1% staining did not show the clustered pattern of CD8^{+} T cells. Based on negative control, we adopted a tentative cut-off point of 0.1% for the HTLV-1/HLA tetramer assay, which was the lower limit of tetramer staining with the HLA-compatible CD8^{+} T cells. Four samples of AC were positive (Fig. 2B, T12, T187, T301, and E11) and the other four were negative (Fig. 2B, T289, T311, E21, and E153) using this cut-off point.

Frequency of anti-HTLV-1 CD8^{+} T cells in PBMC of AC and ATL patients

We assessed a total of 59 subjects consisting of 31 AC patients and 28 ATL patients using eight epitope-specific tetramers per subject possessing HLA-A*0201 or HLA-A*2402. In the case of subjects possessing both HLA alleles (three AC and four ATL), epitope-specific CD8^{+} T cells were analyzed using 16 distinct tetramers per subject.

Frequency of HTLV-1/HLA tetramer positivity varied by HTLV-1 epitope and HLA allele in AC: 90% in Tax_{11-19} with HLA-A*0201, 92% in $Tax_{301-309}$ with HLA-A*2402, and 4–17% in other combinations of Tax_{12-20}, $Tax_{178-185}$, $Tax_{187-195}$, $Tax_{307-315}$, Env_{11-19}, Env_{21-29}, and $Env_{153-161}$ with respective HLA alleles (Table II). In contrast, ATL recognized only Tax_{11-19} with HLA-A*0201 and $Tax_{301-309}$ with HLA-A*2402 at frequencies of 30 and 55%, respectively. Among the individual HTLV-1/HLA tetramers, two (Tax_{11-19}, $p = 0.0042$; and $Tax_{301-309}$, $p = 0.0031$; Table II) were significantly more frequent in AC than ATL. Among AC, 24% of Tax epitopes were positive, whereas significantly fewer epitopes were positive among ATL (9%, $p = 0.0004$; Table II). In contrast, Env epitopes were not statistically significant.

Frequency of subjects detected anti-HTLV-1 CD8^{+} T cells in ATL (15 of 28; 54%) was significantly lower than that in AC (29 of 31; 94%; $p = 0.0003$). In particular, the frequency of subjects possessing Tax-specific CD8^{+} T cells in ATL (15 of 28; 54%) was significantly lower than that in AC (28 of 31; 90%; $p = 0.001$), but differences in frequency possessing Env-specific CD8^{+} T cells were not significant (Table III).

With regard to Tax_{11-19}-specific tetramer binding cells in individual subjects with HLA-A*0201, the frequency of percentage of CD8^{+} T cells binding Tax_{11-19}/HLA-A*0201 tetramer in

Table III. *Summary of HTLV-1-specific tetramer and HTLV-1 proviral load in AC and ATL patients*

	AC	ATL
Subjects positive for Tax tetramer[a]	28 ($n = 31$)	15 ($n = 28$)*
Subjects positive for Env tetramer[a]	4 ($n = 31$)	0 ($n = 28$)
Tax_{11-19} tetramer^{+} CD8^{+} T cells[b]	0.91 ± 0.37 ($n = 10$)	0.74 ± 0.49 ($n = 10$)**
$Tax_{301-309}$ tetramer^{+} CD8^{+} T cells[b]	2.46 ± 0.71 ($n = 24$)	0.21 ± 0.05 ($n = 22$)*
HTLV-1 proviral load	65.4 ± 7.4 ($n = 35$)	1095.4 ± 194.1 ($n = 29$)

[a] The number of subjects positive for tetramers; the percentages of HTLV-1/HLA tetramer^{+} CD8^{+} T cells in the CD8^{+} CD45^{+} T lymphocytes ≥0.1% are counted as subjects positives for tetramer.

[b] The Tax-specific tetramer-positive CD8^{+} T cells in the CD8^{+} CD45^{+} T lymphocytes is shown as the mean ± SE percentage.

[c] The HTLV-1 proviral load is shown as the ±SE copies/10^3 PBMC.

*, $p < 0.0001$; **, $p < 0.05$.

Table IV. *Relationship between HTLV-1 proviral load and HTLV-1 Tax-specific CD8⁺ T cell*

	HTLV-1 Tax-Specific CD8⁺ T Cell			
	Tax_{11-19} Tetramer*		$Tax_{301-309}$ Tetramer	
	Positive ($n = 9$)[b]	Negative ($n = 26$)	Positive ($n = 22$)[b]	Negative ($n = 13$)
Proviral load[a]	38.1 ± 13.8	74.8 ± 8.0	72.4 ± 9.4	53.5 ± 11.6

[a] The HTLV-1 proviral load is shown as the mean ± SE copies/10³ PBMC.
[b] The percentages of HTLV-1/HLA tetramer⁺ CD8⁺ T cells in the CD8⁺ CD45⁺ T lymphocytes that are ≥0.1% are counted as positives.

*, $p < 0.05$, significant differences between positive group and negative group by Mann-Whitney U test.

CD8⁺CD45⁺ T lymphocytes ranged from 0.03 to 3.77% in AC and 0 to 4.43% in ATL patients. There were significant differences in percentage of cells binding Tax_{11-19}/HLA-A*0201 tetramer between AC and ATL ($p = 0.037$; Table III) as well as the frequencies of epitopes found on anti-HTLV-1 CD8⁺ T cells mentioned above. There were also significant differences in percentage of CD8⁺ T cells binding CMV/HLA-A*0201 tetramer in CD8⁺/CD45 T lymphocytes between AC and ATL ($p = 0.028$). With regard to the ratio of $Tax_{301-309}$-specific CD8⁺ T cells in individual subjects with HLA-A*2402, the frequency of percentage of CD8⁺ T cells binding $Tax_{301-309}$/HLA-A*2402 tetramer in CD8/CD45⁺ T lymphocytes ranged from 0 to 15.6% in AC and 0 to 0.79% in ATL patients. There was a significant difference in the ratio of cells binding $Tax_{301-309}$/HLA-A*2402 tetramer between AC and ATL ($p < 0.0001$; Table III) as well as the frequencies of epitopes.

HTLV-1 proviral load in AC and ATL patients

The proviral load of AC and ATL patients ranged from 4.6 to 225.8 and from 44.3 to 2838.3 (copies/10³ PBMC) (Table III), respectively. Because the ATL patients should contain leukemic cells, we assessed the proviral load of only AC in terms of the relationship with Tax-specific tetramer⁺ cells. The proviral load of Tax_{11-19} tetramer-positive AC was significantly lower than that of Tax_{11-19} tetramer-negative AC (mean ± SE, 38.1 ± 13.8 vs 74.8 ± 8.0; $p = 0.043$; Table IV). These findings are consistent with Bangham's report (47) and suggest that Tax_{11-19} CTL works as a strong down-regulator of the proviral load. In contrast, with regard to $Tax_{301-309}$ tetramer, there was no significant difference between the proviral load of $Tax_{301-309}$ tetramer-positive AC and negative AC (mean ± SE, 72.4 ± 9.4 vs 53.5 ± 11.6; Table IV). The reason why the proviral load of $Tax_{301-309}$ tetramer-positive AC was lower than that of negative AC can be explained by the negative group containing many Tax_{11-19} tetramer-positive AC (7 of 13). Therefore, we omitted HLA-A*02-positive (including HLA-A*0201 and HLA-A*0206) subjects in analysis of $Tax_{301-309}$ tetramer. Under this condition, the proviral load of $Tax_{301-309}$ tetramer-positive AC and negative AC was 74.3 ± 10.1 ($n = 18$) and 97.6 ± 11.5 ($n = 5$), respectively ($p = 0.09$).

Expansion of anti-HTLV-1 CD8⁺ T cells by in vitro cultivation

Thirty of 67 subjects (18 AC and 12 ATL patients) were cultured for further analysis of anti-HTLV-1 CD8⁺ T cells in vitro. The cultured cells were morphologically activated T cells and clustered in colony formation. We observed increases in the numbers of positive cells corresponding to Tax_{11-19} in ATL patient and AC (Fig. 3, A and B). Two specificities of HTLV-1 Tax-specific CD8⁺ T cells for $Tax_{289-295}$ and $Tax_{311-319}$ with HLA-A*2402 were newly identified in the cultured PBMC from AC but not from ATL patients (Fig. 3).

Diversity of anti-HTLV-1 CD8⁺ T cells in PBMC of AC and ATL patients

Anti-HTLV-1 CD8⁺ T cells in AC recognized a wide spectrum of Tax and Env epitopes, HLA-A*0201-restricted Tax_{11-19}, $Tax_{178-196}$, $Tax_{307-315}$, and HLA-A*2402-restricted Tax_{12-20}, $Tax_{187-195}$, $Tax_{289-297}$, $Tax_{301-309}$, $Tax_{311-319}$, Env_{11-19}, Env_{21-29}, and $Env_{153-161}$, in vivo or in vitro. In contrast, anti-HTLV-1 CD8⁺ T cells in ATL patients recognized only two epitopes (Tax_{11-19} with HLA-A*0201 and $Tax_{301-309}$ with HLA-A*2402). The number of epitope repertoires found on anti-HTLV-1 CD8⁺ T cells in ATL patients was considerably lower than that in AC (2 of 18 and 11 of 18; $p < 0.05$) as shown in Table III and Fig. 3.
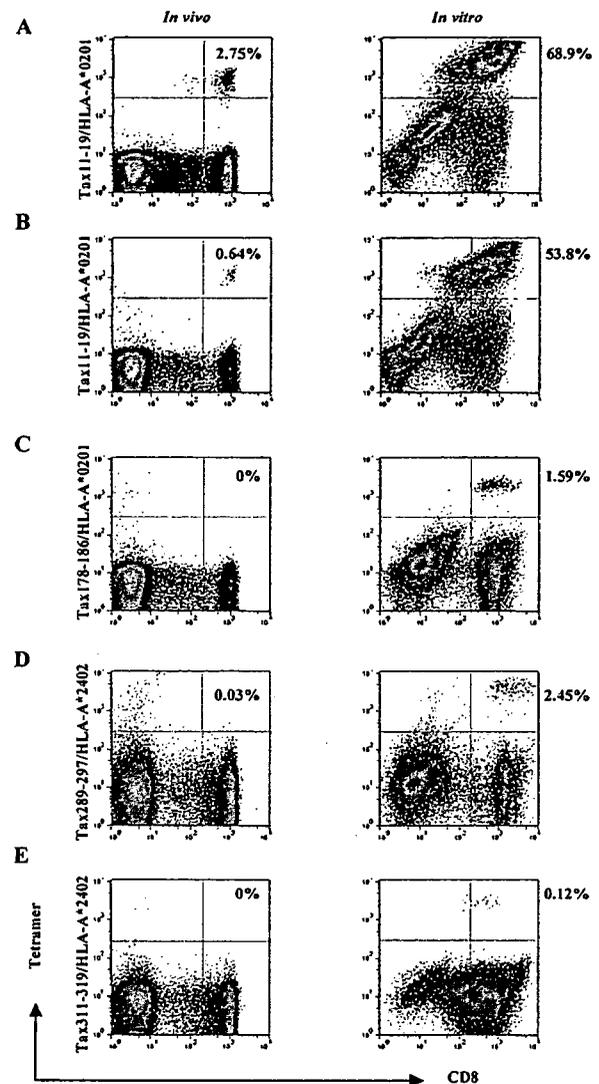

FIGURE 3. In vitro expansion of anti-HTLV-1 CD8⁺ T cells. Freshly isolated PBMC were stained with Tax_{11-19} (A and B), $Tax_{178-186}$ (C), $Tax_{289-297}$ (D), and $Tax_{311-319}$ (E) tetramers, respectively, shown in the *left panels*. Following cultivation with 2×10^{-6} M of each epitope peptide for 14 (A and B), 31 (C), 46 (D), and 22 (E) days, these cells were stained with the respective tetramers, indicated in the *right panels*. Samples of C and D were restimulated after 14 days. Numbers in the *upper right quadrants* represent the percentages of tetramer⁺CD8⁺ T cells in 7-AAD-negative CD8⁺ T lymphocytes. We observed increases in number of positive cells corresponding to peptides.

Intracellular IFN-γ produced in response to HTLV-1 Tax peptide, and expression of perforin and granzyme B

Representative results regarding intracellular cytokines are shown in Fig. 4. Intracellular cytokine⁺tetramer⁺ cell were estimated in CD8⁺ T lymphocytes (R4). None of anti-HTLV-1 CD8⁺ T cells produced IFN-γ in short-term culture without Tax peptide (Fig. 4B, left). IFN-γ production in AC increased from 0 to 52% in the tetramer⁺CD8⁺ T cells (R6/R5+R6) after Tax peptide stimulation (Fig. 4B, upper quadrants of AC, 0/(5.8 + 0) vs 1.1/(1.0 + 1.1)). Similarly, that in ATL patient increased from 0 to 40% in the tetramer⁺CD8⁺ T cells after Tax peptide stimulation (Fig. 2B, upper quadrants of ATL, 0/(1.0 + 0) vs 0.4/(0.6 + 0.4)). Intracellular IFN-γ production was also detected in PBMC of all subjects examined (5 of both AC and ATL patients; average ratios of positive cells were 21.8 and 13.1%, respectively). Thus, production of IFN-γ demonstrated that the tetramer⁺CD8⁺ T cells in AC and ATL patients are functional CD8⁺ T cells targeting HTLV-1 Tax epitopes. Tax peptide stimulation caused decrease of

tetramer⁺CD8⁺ T cell. After peptide stimulation, CD8⁺7-AAD⁺ cells were increased more than untreated culture. Expression of the degranulation marker CD107a, which allows measurement of cytolytic cell activation (28, 45, 46), was significantly increased in tetramer⁺CD8⁺ lymphocytes treated with the peptide (Fig. 4C). These findings suggest that the tetramer⁺CD8⁺ cells were decreased as a result of cytotoxicity.

Intracellular perforin and granzyme B were detected in HTLV-1 Tax tetramer⁺CD8⁺ T lymphocytes of all subjects examined (10 of both AC and ATL patients, respectively; representative data are shown in Fig. 4, D and E). AC subject had 57% (R6/R5+R6) of perforin⁺ cells and 40% of granzyme B⁺ cells in HTLV-1 Tax tetramer⁺CD8⁺ T lymphocytes (shown in upper column of Fig. 4, D and E, respectively), whereas ATL subject had 30% of perforin⁺ cells and 36% of granzyme B⁺ cells (shown in upper column of Fig. 4, D and E, respectively).

Interestingly, notable insufficiency of perforin (Fig. 5A) and granzyme B (Fig. 5B) expression in HTLV-1 Tax tetramer⁺CD8⁺
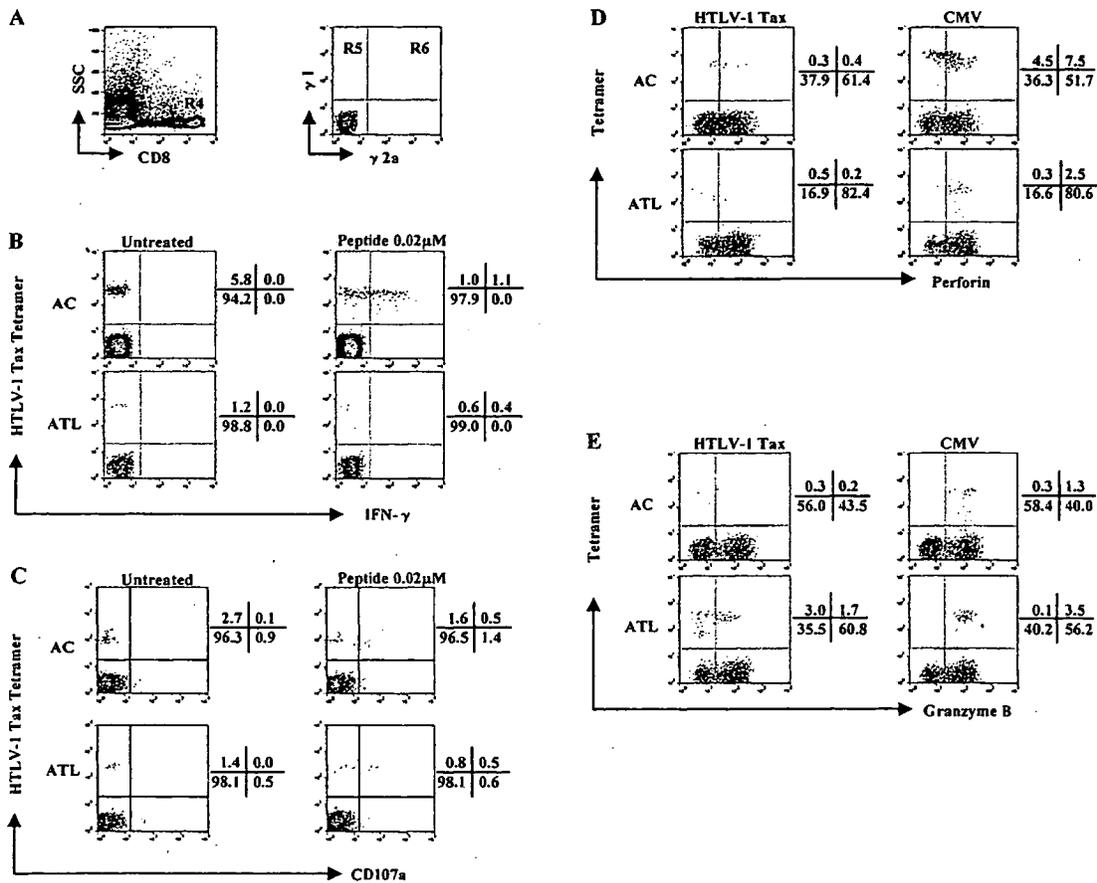

FIGURE 4. Intracellular production of IFN-γ, CD107a mobilization, and expression of perforin and granzyme B in HTLV-1-specific CD8⁺ T lymphocytes. A, Intracellular cytokine⁺tetramer⁺ cell were estimated in CD8⁺ T lymphocytes (R4). The right panel, which was extended in the R4 region, showed negative control for surface and intracellular immunofluorescence. B, Intracellular IFN-γ production in anti-HTLV-1 CD8⁺ T cells are shown on the top (AC) and bottom (ATL), respectively. The left panel showed untreated condition, whereas the right panel showed <0.02 μM peptide concentration. Increases of IFN-γ were observed corresponding to peptide pulsing in AC and ATL patient. Numbers indicate the percentages in CD8⁺ T lymphocytes. Figure shows one representative result of IFN-γ production in five of both AC and ATL patients. C, CD107a mobilization in anti-HTLV-1 CD8⁺ T cells are shown on the top (AC) and bottom (ATL), respectively. The left panel showed untreated condition, whereas the right panel showed <0.02 μM peptide concentration. Increases of CD107a⁺tetramer⁺ cells were observed corresponding to peptide pulsing in AC and ATL patient. Figure shows one representative result of CD107a mobilization assay in three of both AC and ATL patients, respectively. D, Intracellular perforin expression in tetramer⁺CD8⁺ T cells are shown on the top (AC) and bottom (ATL), respectively. The left panel showed anti-HTLV-1 Tax CD8⁺ T cells, whereas the right panel showed anti-CMV CD8⁺ T cells. Figure shows 1 representative result of granzyme B expression in 10 of both AC and ATL patients, respectively. E, Intracellular granzyme B expression in tetramer⁺CD8⁺ T cells are shown on the top (AC) and bottom (ATL), respectively. The left panel showed anti-HTLV-1 Tax CD8⁺ T cells, whereas the right panel showed anti-CMV CD8⁺ T cells. Figure shows 1 representative result of granzyme B expression in 10 of both AC and ATL patients, respectively.
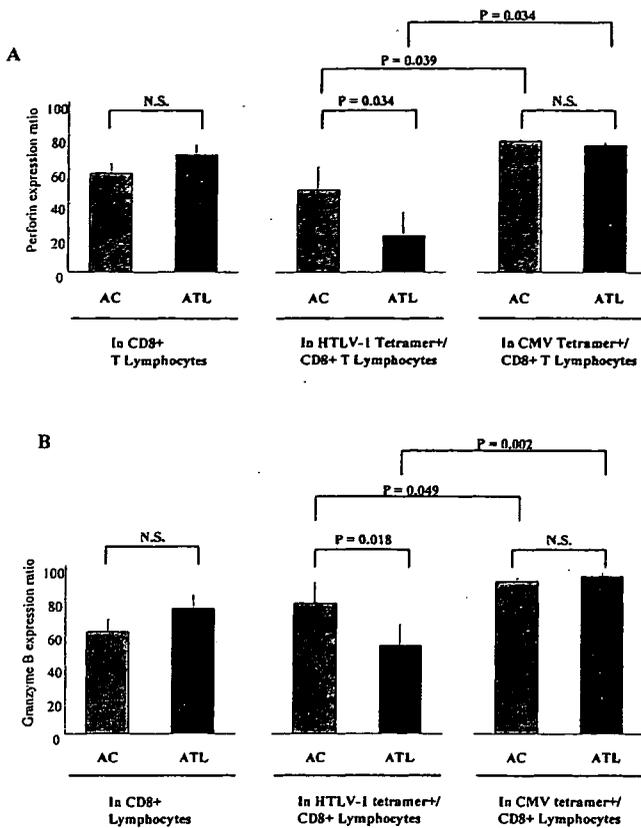
A

B

FIGURE 5. Differential expression of perforin and granzyme B in anti-HTLV-1 and anti-CMV CD8$^+$ T lymphocytes between AC and ATL. *A*, Expression of perforin in CD8$^+$, HTLV-1 Tax tetramer$^+$CD8$^+$, and CMV tetramer$^+$CD8$^+$ T lymphocytes (mean of 10 independent experiments). *B*, Expression of granzyme B in CD8$^+$, HTLV-1 Tax tetramer$^+$CD8$^+$ and CMV tetramer$^+$CD8$^+$ T lymphocytes (mean of 10 independent experiments).

T lymphocytes were observed in ATL in comparison with AC, but not in CMV tetramer$^+$CD8$^+$ T lymphocytes. Regarding perforin, expression in anti-Tax CTL of ATL were significantly lower than AC (average ratios of positive cells were 21.7 and 48.2%, respectively; $p = 0.034$), but not in CD8$^+$ T lymphocytes. Regarding granzyme B, expression in anti-Tax CTL of ATL were also significantly lower than AC (average ratios of positive cells were 52.5 and 77.6%, respectively; $p = 0.018$), but not in CD8$^+$ T lymphocytes.

In addition, expression of perforin and granzyme B in HTLV-1 Tax tetramer$^+$CD8$^+$ T lymphocytes were diminished in comparison with those in CMV tetramer$^+$CD8$^+$ T lymphocytes in both AC (anti-HTLV-1 vs anti-CMV with perforin and granzyme B; $p = 0.039$ and $p = 0.049$, respectively) and ATL (anti-HTLV-1 vs anti-CMV with perforin and granzyme B; $p = 0.034$ and $p = 0.002$, respectively).

Discussion

HTLV-1-specific CTL are thought to be important immune effectors that suppress the outgrowth of HTLV-1-transformed T cell and thus reduce the risk of ATL development (9–14, 21). To confirm the correlation between a deficiency of anti-HTLV-1 CTL and increased risk of ATL, we compared the frequency of anti-HTLV-1 CTL and diversity of epitope in freshly isolated PBMC from AC and ATL patients using the tetramers. Our results demonstrated that the frequency and diversity of anti-HTLV-1 Tax CD8$^+$ T cells in ATL patients was significantly reduced compared

with those in AC. These observations suggest that the lower frequency and diversity of anti-Tax CD8$^+$ T cells is risk for ATL development. A recent report demonstrated insufficient expression of HTLV-1 Tax in vivo in ATL patients and suspected a role of Tax-specific CTL for therapy or prevention of ATL (48). However, other researchers, as well as our group, demonstrated clear expression of HTLV-1 Tax in short-term cultures of fresh ATL cells (24–26). It is likely that cell-to-cell interactions in short-term culture mimic the cellular interactions in lymphoid tissues in vivo. Therefore, it is possible that ATL cells in lymphoid tissues produce HTLV-1 Tax protein. In fact, Marin et al. (49) recently demonstrated HTLV-1 Tax expression by immunohistochemistry in lymphoid tissues in ATL patients. Regarding AC, Hanon et al. (12) also demonstrated HTLV-1 Tax expression in cultured PBMC from HTLV-1-infected carriers. Although direct evidence that infected cells in lymphoid tissue produce HTLV-1 Tax in vivo has not been reported, it is likely that the cells produce Tax protein similar to primary ATL cells. A few previous studies that demonstrated CTL activity stimulated by Tax peptide have been reported (14, 50). However they did not indicate quantitative anti-Tax CD8$^+$ T cells. The present studies examined directly for anti-Tax CD8$^+$ T cells in vivo of AC and ATL patients by tetramer assay for quantitative analysis.

We identified 11 of 16 distinct clones of anti-HTLV-1 CD8$^+$ T cells in the PBMC of AC and 2 clones of CD8$^+$ T cells in ATL patients (Table III and Fig. 3). The CD8$^+$ T cells for Tax_{11-19} and $Tax_{301-309}$ were commonly detected in AC and ATL patients carrying HLA-A*0201 or HLA-A*2402, although the detection rate in ATL patients was much lower than that in AC. Therefore, Tax_{11-19} and $Tax_{301-309}$ were thought to be the major epitopes for Tax-specific CD8$^+$ T cell in both AC and ATL patients. In fact, $Tax_{301-309}$ was found to be the immunodominant epitope for anti-Tax CTL generated in ATL patients with HLA-A*2402 who underwent allogeneic hemopoietic stem cell transplantation (20). Nine other epitopes, Tax_{12-20}, $Tax_{178-186}$, $Tax_{187-195}$, $Tax_{289-297}$, $Tax_{309-315}$, $Tax_{311-319}$, Env_{11-19}, Env_{21-29}, and $Env_{153-161}$, were likely to be minor epitopes for generation of anti-HTLV-1 CD8$^+$ T cells. It is possible that even minor epitopes have significant immune function in vivo, although we have no direct evidence.

In the present study, we showed that anti-Tax CD8$^+$ T cells are significantly more abundant in patients with AC compared with ATL patients, but anti-Env is not in both HLA phenotypes. These findings suggest strongly that anti-Tax CD8$^+$ T cells are more significant for prevention of the development of ATL than anti-Env CD8$^+$ T cells. High levels of Tax-specific CD8$^+$ T cells are advantageous for suppression of outgrowth of HTLV-1-infected or -transformed T cell and thus reduce the risk of ATL (9–14, 21). The present study demonstrated that the Tax_{11-19}-specific tetramer$^+$CD8$^+$ T cells works as a strong down-regulator of the proviral load (47). The $Tax_{301-309}$-specific tetramer$^+$CD8$^+$ T cells show the tendency of decreasing the viral load. This interpretation is supported by the results of our previous study in which risk of ATL was shown to be associated with the number of HLA anchor motifs that recognized HTLV-1 Tax epitopes but not HTLV-1 Env epitopes (19). If deficient anti-Tax CD8$^+$ T cells are responsible for the development of ATL, AC with low frequency or diversity of anti-Tax CD8$^+$ T cells may belong to a group at high risk of developing ATL. Further follow-up studies are needed to clarify the significance of anti-Tax CD8$^+$ T cells for prevention of this disease.

ATL patients usually show immune dysfunction, and this may explain the lower frequency of CTL in these subjects. In fact, dysfunction of cellular immunity has been reported since the discovery of this disease (32). Therefore, the low frequency of

anti-Tax CTL may be just one of the general immune dysfunctions present in ATL patients. Conversely, the low frequency of anti-HTLV-1 CTL may be an Ag-specific phenomenon, and not representative of whole immune dysfunction. Our observation of low anti-Tax CD8$^+$ T cells frequency despite high numbers of CD8$^+$ cells in ATL patients (T11, 0.05%; T301, 0.01%, 663 cells/μl; T301, 0.12%, 550 cells/μl) and high anti-Tax CD8$^+$ T cells frequency despite low numbers of CD8$^+$ T cells in AC (T11, 0.64%, 356 cells/μl; T301, 1.16%, 309 cells/μl) may support the latter suggestion (normal values are between 400 and 800 CD8$^+$ T cells/μl). In such cases, the low frequency of anti-Tax CD8$^+$ T cells is likely to be Ag-specific immune dysfunction rather than general immune dysfunction. The low frequency of anti-Tax CD8$^+$ T cells in ATL patients may be involved in progression from HTLV-1 carrier to ATL, and also contribute to the aggressiveness of this disease, which is refractory to treatment.

The present study demonstrated that HTLV-1 Tax tetramer$^+$CD8$^+$ T cells in AC and ATL patients produce intracellular IFN-γ, and possess perforin and granzyme B, which are molecular markers of functional CTL in response to the corresponding peptides. Interestingly, perforin and granzyme B expressions in Tax-specific tetramer-positive cells are significantly lower in ATL than AC, but there was no difference in CMV-specific tetramer-positive cells. These findings suggest that reduction of Tax-specific tetramer positive cells in not only frequency and diversity but also the function work as risk for ATL development. In contrast, the present study demonstrated that the reduction in CMV-specific tetramer-positive cells CTL is observed only in the frequency but not in the function. Therefore, frequent CMV infection during clinical course of ATL patient may be caused by this mechanism. In contrast, the present study demonstrated that the two functional molecules expression is reduced in anti-Tax CTL but not in anti-CMV CTL in either AC subjects or ATL patients. Although the mechanism why such differential regulation of CTL function with Ag specificity works in AC or ATL is unclear at present, these findings suggest that dysfunction of anti-Tax CTL in the present study reflect Ag specificity but not general immune function.

In conclusion, our HTLV-1/HLA tetramer assay enabled analysis of anti-HTLV-1 CD8$^+$ T cells in PBMC of AC and ATL patients and demonstrated deletion of anti-Tax CD8$^+$ T cells in ATL patients. Intracellular cytokine expression in anti-HTLV-1 CD8$^+$ T cells had significant difference between AC and ATL, but not in anti-CMV CD8$^+$ T cells. The reduced frequency, diversity, and function of anti-HTLV-1 Tax CD8$^+$ T cell clones may be related to the development of ATL. This HLA tetramer assay can be used for monitoring the in vivo status of CTL, and it may be possible to identify the high risk group in AC of developing to ATL. Furthermore, the successful expansion of anti-Tax CTL clones in the present study may facilitate the development of novel approaches for immunoadaptive therapy against ATL.

Acknowledgments

Disclosures

The authors have no financial conflict of interest.

References

1. Uchiyama, T., J. Yodoi, K. Sagawa, K. Takatsuki, and H. Uchino. 1977. Adult T cell leukemia: clinical and hematologic features of 16 cases. Blood 50: 481–492.
2. Popovic, M., M. S. Reitz, Jr., M. G. Samgadharan, M. Robert-Guroff, V. S. Kalyanaraman, Y. Nakao, I. Miyoshi, J. Minowada, M. Yoshida, Y. Ito, and

R. C. Gallo. 1982. The virus of Japanese adult T-cell leukaemia is a member of the human T-cell leukaemia virus group. Nature 300: 63–66.
3. Yoshida, M., I. Miyoshi, and Y. Hinuma. 1982. Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease. Proc. Natl. Acad. Sci. USA 79: 2031–2035.
4. de The, G., and R. Bomford. 1993. An HTLV-I vaccine: why, how, for whom? AIDS Res. Hum. Retroviruses 9: 381–386.
5. Tajima, K. 1990. The 4th nation-wide study of adult T-cell leukemia/lymphoma (ATL) in Japan: estimates of risk of ATL and its geographical and clinical features: the T- and B-Cell Malignancy Study Group. Int. J. Cancer 15: 237–243.
6. Yamada, Y., M. Tomonaga, H. Fukuda, S. Hanada, A. Utsunomiya, M. Tara, M. Sano, S. Ikeda, K. Takatsuki, M. Kozuru, et al. 2001. A new G-CSF-supported combination chemotherapy, LSG15, for adult T-cell leukaemia-lymphoma: Japan Clinical Oncology Group Study 9303. Br. J. Haematol. 113: 375–382.
7. Borg, A., J. A. Yin, P. R. Johnson, J. Tosswill, M. Saunders, and D. Morris. 1996. Successful treatment of HTLV-1-associated acute adult T-cell leukaemia lymphoma by allogeneic bone marrow transplantation. Br. J. Haematol. 94: 713–715.
8. Utsunomiya, A., Y. Miyazaki, Y. Takatsuka, S. Hanada, K. Uozumi, S. Yashiki, M. Tara, F. Kawano, Y. Saburi, H. Kikuchi, et al. 2001. Improved outcome of adult T cell leukemia/lymphoma with allogeneic hematopoietic stem cell transplantation. Bone Marrow Transplant. 27: 15–20.
9. Jacobson, S., H. Shida, D. E. McFarlin, A. S. Fauci, and S. Koenig. 1990. Circulating CD8$^+$ cytotoxic T lymphocytes specific for HTLV-I pX in patients with HTLV-I associated neurological disease. Nature 348: 245–248.
10. Mitsuya, H., L. A. Matis, M. Megson, P. A. Bunn, C. Murray, D. L. Mann, R. C. Gallo, and S. Broder. 1983. Generation of an HLA-restricted cytoxic T cells reactive against cultured tumor cells from a patient infected with human T cell leukemia/lymphoma virus. J. Exp. Med. 158: 994–999.
11. Kannagi, M., S. Harada, I. Maruyama, H. Inoko, H. Igarashi, G. Kuwashima, S. Sato, M. Morita, M. Kidokoro, M. Sugimoto, et al. 1991. Predominant recognition of human T cell leukemia virus type I (HTLV-I) pX gene products by human CD8$^+$ cytotoxic T cells directed against HTLV-I-infected cells. Int. Immunol. 3: 761–767.
12. Hanon, E., S. Hall, G. P. Taylor, M. Saito, R. Davis, Y. Tanaka, K. Usuku, M. Osame, J. N. Weber, and C. R. Bangham. 2000. Abundant tax protein expression in CD4$^+$ T cells infected with human T-cell lymphotropic virus type I (HTLV-I) is prevented by cytotoxic T lymphocytes. Blood 95: 1386–1392.
13. Kannagi, M., N. Harashima, K. Kurihara, T. Ohashi, A. Utsunomiya, R. Tanosaki, M. Masuda, M. Tomonaga, and J. Okamura. 2005. Tumor immunity against adult T-cell leukemia. Cancer Sci. 96: 249–255.
14. Arnulf, B., M. Thorel, Y. Poirot, R. Tamouza, E. Boulanger, A. Jaccard, E. Oksenhendler, O. Hermine, and C. Pique. 2004. Loss of the ex vivo but not the reinducible CD8$^+$ T-cell response to Tax in human T-cell leukemia virus type I-infected patients with adult T-cell leukemia/lymphoma. Leukemia 18: 126–132.
15. Uchiyama, T. 1997. Human T cell leukemia virus type I (HTLV-I) and human diseases. Ann. Rev. Immunol. 15: 15–37.
16. Usuku, K., S. Sonoda, M. Osame, S. Yashiki, K. Takahashi, M. Matsumoto, T. Sawada, K. Tsuji, M. Tara, and A. Igata. 1988. HLA haplotype-linked high immune responsiveness against HTLV-I in HTLV-I-associated myelopathy: comparison with adult T-cell leukemia/lymphoma. Ann. Neurol. 23(Suppl.): S143–S150.
17. Motomura, M., T. Nakamura, K. Nagasato, K. Shibayama, S. Kubo, I. Nakasono, M. Tsujihata, S. Sonoda, and S. Yashiki. 1990. HTLV-I associated myelopathy in an identical twin. Lancet 336: 55.
18. Sonoda, S., T. Fujiyoshi, and S. Yashiki. 1996. Immunogenetics of HTLV-I/II and associated diseases. J. Acquir. Immune. Defic. Syndr. Hum. Retrovirol. 13(Suppl. 1): S119–S123.
19. Yashiki, S., T. Fujiyoshi, N. Arima, M. Osame, M. Yoshinaga, Y. Nagata, M. Tara, K. Nomura, A. Utsunomiya, S. Hanada, et al. 2001. HLA-A*26, HLA-B*4002, HLA-B*4006, and HLA-B*4801 alleles predispose to adult T cell leukemia: the limited recognition of HTLV type I tax peptide anchor motifs and epitopes to generate anti-HTLV type 1 tax CD8$^+$ cytotoxic T lymphocytes. AIDS Res. Hum. Retroviruses 17: 1047–1061.
20. Harashima, N., K. Kurihara, A. Utsunomiya, R. Tanosaki, S. Hanabuchi, M. Masuda, T. Ohashi, F. Fukui, A. Hasegawa, T. Masuda, et al. 2004. Graft-versus-Tax response in adult T-cell leukemia patients after hematopoietic stem cell transplantation. Cancer Res. 64: 391–399.
21. Parker, C. E., S. Nightingale, G. P. Taylor, J. Weber, and C. R. Bangham. 1994. Circulating anti-Tax cytotoxic T lymphocytes from human T-cell leukemia virus type I-infected people, with and without tropical spastic paraparesis, recognize multiple epitopes simultaneously. J. Virol. 68: 2860–2868.
22. Freedman, L. R., J. C. Cerottini, and K. T. Brunner. 1972. In vivo studies of the role of cytotoxic T-cells in tumor allograft immunity. J. Immunol. 109: 1371–1378.
23. Roden, M. M., K. H. Lee, M. C. Panelli, and F. M. Marincola. 1999. A novel cytolysis assay using fluorescent labeling and quantitative fluorescent scanning technology. J. Immunol. Methods 226: 29–41.
24. Setoyama, M., Y. Katahira, T. Hamada, M. Tashiro, S. Yashiki, Y. Tanaka, H. Tozawa, and S. Sonoda. 1992. Expression of human T-cell lymphotropic virus type-1 gene products in the short-term cultured skin tissues of an adult T-cell leukemia/lymphoma patient with cutaneous manifestations. J. Dermatol. 19: 133–139.
25. Arima, N., K. Matsushita, H. Obata, H. Ohtsubo, H. Fujiwara, K. Arimura, T. Kukita, Y. Suruga, S. Wakamatsu, S. Hidaka, and C. Tei. 1999. NF-κB involvement in the activation of primary adult T-cell leukemia cells and its clinical implications. Exp. Hematol. 27: 1168–1175.

26. Yoshida, M. 2001. Multiple viral strategies of HTLV-1 for dysregulation of cell growth control. *Annu. Rev. Immunol.* 19: 475–496.

27. Harty, J. T., A. R. Tvinnereim, and D. W. White. 2000. CD8$^+$ T cell effector mechanisms in resistance to infection. *Annu. Rev. Immunol.* 18: 275–308.

28. Betts, M. R., D. A. Price, J. M. Brenchley, K. Lore, F. J. Guenaga, A. Smed-Sorensen, D. R. Ambrozak, S. A. Migueles, M. Connors, M. Roederer, et al. 2004. The functional profile of primary human antiviral CD8$^+$ T cell effector activity is dictated by cognate peptide concentration. *J. Immunol.* 172: 6407–6417.

29. Lim, D. G., B. K. Bieganowska, G. J. Freeman, and D. A. Hafler. 1999. Direct analysis of viral-specific CD8$^+$ T cells with soluble HLA-A2/Tax$_{11–19}$ tetramer complexes in patients with human T cell lymphotropic virus-associated myelopathy. *J. Immunol.* 162: 1765–1771.

30. Tomaru, U., Y. Yamano, M. Nagai, D. Maric, P. T. Kaumaya, W. Biddison, and S. Jacobson. 2003. Detection of virus-specific T cells and CD8$^+$ T-cell epitopes by acquisition of peptide-HLA-GFP complexes: analysis of T-cell phenotype and function in chronic viral infections. *Nat. Med.* 9: 469–476.

31. Sandberg, J. K., N. M. Fast, and D. F. Nixon. 2001. Functional heterogeneity of cytokines and cytolytic effector molecules in human CD8$^+$ T lymphocytes. *J. Immunol.* 167: 181–187.

32. Shimoyama, M. 1991. Diagnostic criteria and classification of clinical subtypes of adult T-cell leukaemia-lymphoma: a report from the Lymphoma Study Group (1984–87). *Br. J. Haematol.* 79: 428–437.

33. Osame, M., M. Nakagawa, F. Umehara, S. Ijichi, T. Moritoyo, I. Higuchi, K. Usuku, K. Arimura, and S. Izumo. 1997. Recent studies on the epidemiology, clinical features and pathogenic mechanisms of HTLV-I associated myelopathy (HAM/TSP) and other diseases associated to HTLV. *J. Neurovirol.* 3(Suppl. 1): S50–S51.

34. Sonoda, S. 2003. Genetic risk of disease development among HTLV-1 carriers. *Gann Monogr. Cancer Res.* 50: 289–301.

35. Katahira, Y., S. Yashiki, T. Fujiyoshi, K. Nomura, M. Tara, M. Mori, M. Setoyama, T. Kanzaki, H. Shida, and S. Sonoda. 1995. In vitro induction of cytotoxic T lymphocytes against HTLV-I-infected T-cells from adult T-cell leukemia patients, asymptomatic HTLV-I carriers and seronegative healthy donors. *Jpn. J. Cancer Res.* 86: 21–27.

36. Itoh, Y., N. Mizuki, T. Shimada, F. Azuma, M. Itakura, K. Kashiwase, E. Kikkawa, J. K. Kulski, M. Satake, and H. Inoko. 2005. High-throughput DNA typing of HLA-A, -B, -C, and -DRB1 loci by a PCR-SSOP-Luminex method in the Japanese population. *Immunogenetics* 57: 717–729.

37. Altman, J. D., P. A. Moss, P. J. Goulder, D. H. Barouch, M. G. McHeyzer-Williams, J. I. Bell, A. J. McMichael, and M. M. Davis. 1996. Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274: 94–96.

38. Bodinier, M., M. A. Peyrat, C. Tournay, F. Davodeau, F. Romagne, M. Bonneville, and F. Lang. 2000. Efficient detection and immunomagnetic sorting of specific T cells using multimers of MHC class I and peptide with reduced CD8 binding. *Nat. Med.* 6: 707–710.

39. Haanen, J. B., M. G. van Oijen, F. Tirion, L. C. Oomen, A. M. Kruisbeek, F. A. Vyth-Dreese, and T. N. Schumacher. 2000. In situ detection of virus- and tumor-specific T-cell immunity. *Nat. Med.* 6: 1056–1060.

40. Skinner, P. J., M. A. Daniels, C. S. Schmidt, S. C. Jameson, and A. T. Haase. 2000. Cutting edge: in situ tetramer staining of antigen-specific T cells in tissues. *J. Immunol.* 165: 613–617.

41. Kuzushima, K., N. Hayashi, H. Kimura, and T. Tsurumi. 2001. Efficient identification of HLA-A*2402 restricted cytomegalovirus-specific CD8$^+$ T-cell epitopes by a computer algorithm and an enzyme-linked immunospot assay. *Blood* 98: 1872–1881.

42. Lecoeur, H., E. Ledru, M. C. Prevost, and M. L. Gougeon. 1997. Strategies for phenotyping apoptotic peripheral human lymphocytes comparing ISNT, annexin-V and 7-AAD cytofluorometric staining methods. *J. Immunol. Methods* 209: 111–123.

43. Sonoda, J., C. Koriyama, S. Yamamoto, T. Kozako, H. C. Li, C. Lema, S. Yashiki, T. Fujiyoshi, M. Yoshinaga, Y. Nagata, et al. 2004. HTLV-1 provirus load in peripheral blood lymphocytes of HTLV-1 carriers in diminished by green tea drinking. *Cancer Sci.* 95: 596–601.

44. Prussin, C., and D. D. Metcalfe. 1995. Detection of intracytoplasmic cytokine using flow cytometry and directly conjugated anti-cytokine antibodies. *J. Immunol. Methods* 188: 117–128.

45. Rubio, V., T. B. Stuge, N. Singh, M. R. Betts, J. S. Weber, M. Roederer, and P. P. Lee. 2003. Ex vivo identification, isolation and analysis of tumor-cytolytic T cells. *Nat. Med.* 9: 1377–1382.

46. Burkett, M. W., K. A. Shafer-Weaver, S. Strobl, M. Baseler, and A. Malyguine. 2005. A novel flow cytometric assay for evaluating cell-mediated cytotoxicity. *J. Immunother.* 28: 396–402.

47. Bangham, C. R. 2003. The immune control and cell-to-cell spread of human T-lymphotropic virus type 1. *J. Gen. Virol.* 84: 3177–3189.

48. Taylor, G. P., and M. Matsuoka. 2005. Natural history of adult T-cell leukemia/lymphoma and approaches to therapy. *Oncogene* 24: 6047–6057.

49. Marin, O., K. Hasui, C. Remondegui, E. Sato, M. M. Aye, N. Takenouchi, S. Izumo, and K. Tajima. 2002. Adult T-cell leukemia/lymphoma in Jujuy, northwest Argentina. *Pathol. Int.* 52: 348–357.

50. Kubota, R., T. Kawanishi, H. Matsubara, A. Manns, and S. Jacobson. 1998. Demonstration of human T lymphotropic virus type I (HTLV-I) tax-specific CD8$^+$ lymphocytes directly in peripheral blood of HTLV-I-associated myelopathy/tropical spastic paraparesis patients by intracellular cytokine detection. *J. Immunol.* 161: 482–488.

Anti-HTLV-1 Tax Antibody and Tax-Specific Cytotoxic T Lymphocyte Are Associated With a Reduction in HTLV-1 Proviral Load in Asymptomatic Carriers

Masaki Akimoto,[1] Tomohiro Kozako,[2] Takashi Sawada,[3] Kakushi Matsushita,[1] Atsuo Ozaki,[1] Heiichiro Hamada,[1] Hideaki Kawada,[1] Makoto Yoshimitsu,[1] Masahito Tokunaga,[1] Koichi Haraguchi,[1] Kimiharu Uozumi,[2] Naomichi Arima,[2]* and Chuwa Tei[4]

[1]Department of Hematology and Immunology, Kagoshima University Hospital, Kagoshima, Japan
[2]Division of Host Response, Center for Chronic Viral Diseases,
Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan
[3]Clinical Research Center, Eisai Co., Ltd., Tokyo, Japan
[4]Department of Cardiovascular, Respiratory and Metabolic Medicine,
Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan

Previous studies have suggested that higher anti-human T-lymphotropic virus 1 (HTLV-1) antibody titer and lower anti-HTLV-1 Tax antibody reactivity are risk factors for adult T-cell leukemia/lymphoma. In the present study, we analyzed the relationships between these factors and clarified their significance. Forty-five carriers were examined for anti-HTLV-1 and anti-Tax antibody by ELISA. In addition, 43 of the 45 carriers with HLA-A*0201 and/or A*2402 were examined for frequency of Tax-specific cytotoxic T lymphocytes (CTLs) using HTLV-1/HLA tetramers, and 44 were examined for proviral load by real-time PCR. The relationships between these factors were analyzed statistically. The frequencies of Tax11-19 and Tax301-309-specific CTLs were significantly higher in the anti-Tax antibody-positive group as compared with the antibody-negative group ($P = 0.002$ and 0.033, respectively). Anti-HTLV-1 antibody titer had a positive correlation with proviral load ($P = 0.019$), whereas anti-Tax antibody did not show a significant correlation. Higher frequencies of both Tax11-19 and Tax301-309-specific CTLs are related to a reduction in proviral load ($P = 0.017$ and 0.015, respectively). Synergistic interactions of humoral and cellular immunity against Tax protein were demonstrated in HTLV-1 carriers. Tax-specific CTL may reduce HTLV-1 proviral load to prevent asymptomatic carriers from developing adult T-cell leukemia/lymphoma. J. Med. Virol. 79:977–986, 2007.
© 2007 Wiley-Liss, Inc.

KEY WORDS: anti-Tax antibody; CTL; HTLV-1; proviral load; carrier

INTRODUCTION

Human T-lymphotropic virus 1 (HTLV-1) is a strain in the genus Deltaretrovirus, family Retroviridae. It is etiologically linked to adult T-cell leukemia/lymphoma [Poiesz et al., 1980; Hinuma et al., 1981] and a chronic inflammatory neurological disorder designated HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [Gessain et al., 1985; Osame et al., 1986]. HTLV-1 is prevalent in Japan, the Caribbean, South America, Africa, Melanesia, and the Middle East [Maloney and Blattner, 2003]. In Japan, it is estimated that approximately 1 million people are infected with HTLV-1, and most HTLV-1 carriers are asymptomatic throughout their lives. However, 1–5% of infected individuals develop adult T-cell leukemia/lymphoma [Tajima, 1990], and less than 1% develop HAM/TSP or other inflammatory disorders.

Although adult T-cell leukemia/lymphoma is caused by HTLV-1 infection, generally a long latent period about 60 years is needed for the development of the disease after initial infection mainly via breastfeeding [Hino et al., 1985; Kinoshita et al., 1987]. The Miyazaki Cohort Study of HTLV-1 carriers suggested that the

risk factors for the development of adult T-cell leukemia/
lymphoma from carriers include vertical HTLV-1 infec-
tion, gender (male > female), and increased numbers of
abnormal lymphocytes (Ably) [Hisada et al., 1998a,
2001]. Others have reported that the percentage of
circulating Ably are strongly correlated with HTLV-1
proviral load [Hisada et al., 1998a; Tachibana et al.,
1992]. Moreover, persistent proliferation of HTLV-1-
infected CD4$^+$ T-cell clones in asymptomatic carriers is
accompanied with a high proviral load [Etoh et al.,
1997]. These observations suggest that high HTLV-1
proviral load is also correlated with a risk of the
development of adult T-cell leukemia/lymphoma.

Anti-HTLV-1 antibody is detected in all HTLV-1-
infected individuals, either asymptomatic carriers or
patients with HTLV-1-associated diseases. In the
Miyazaki Cohort Study, a higher anti-HTLV-1 antibody
titer was a high risk factor for the development of adult
T-cell leukemia/lymphoma [Hisada et al., 1998b]. In
contrast, a lower prevalence of antibody to Tax protein
(anti-Tax antibody) has been demonstrated in patients
with adult T-cell leukemia/lymphoma compared with
asymptomatic HTLV-1 carriers [Yokota et al., 1989]. A
lower prevalence of anti-Tax antibody has also been
demonstrated in a subset of HTLV-1 carriers with
detectable levels of Ably (>0.6%) [Hisada et al., 1998a],
suggesting that anti-Tax antibody reactivity is low
among patients with adult T-cell leukemia/lymphoma
before their clinical diagnosis. Anti-Tax antibody levels
in all five subjects who developed adult T-cell leukemia/
lymphoma were low for up to 10 years preceding their
diagnosis in the cohort study. These findings suggest
that a lower anti-Tax antibody level is a significant risk
factor for the development of adult T-cell leukemia/
lymphoma [Hisada et al., 1998b].

Recently, HTLV-1-specific cytotoxic T lymphocytes
(CTLs) have attracted a great deal of attention with
regard to host immunity against infection with the
virus. HTLV-1 Tax protein is the most common
target for HTLV-1-specific CTLs in infected individuals
[Jacobson et al., 1990; Kannagi et al., 1991]. Tax-specific
CTLs can be induced from peripheral blood mono-
nuclear cells (PBMCs) of asymptomatic carriers
in vitro. In contrast, induction of Tax-specific CTLs is
rarely observed in PBMCs of patients with adult T-cell
leukemia/lymphoma [Kannagi et al., 1983, 1984, 1993,
1994; Jacobson et al., 1990; Parker et al., 1992]. The
insufficient Tax-specific CTL response in patients with
adult T-cell leukemia/lymphoma suggests that these
CTLs contribute to surveillance for the disease in HTLV-
1-infected individuals.

On the other hand, previous studies have suggested
that HTLV-1 proviral load is also a major risk factor for
HAM/TSP; the median proviral load is 16-fold higher in
HAM/TSP patients than in healthy carriers [Nagai
et al., 1998]. A high HTLV-1 proviral load is also
associated with an increased risk of progression to
disease [Nagai et al., 1998; Taylor et al., 1999].

These findings suggest that a reduction in HTLV-1
proviral load in circulating lymphocytes prevents HTLV-

1 carriers from contracting adult T-cell leukemia/
lymphoma and HAM/TSP. Although anti-HTLV-1 anti-
body, anti-Tax antibody, and Tax-specific CTLs seem to
be related to HTLV-1 proviral load and the development
of HTLV-1-associated diseases, the details are poorly
understood. In the present study, we demonstrated
relationships between anti-HTLV-1 antibody, anti-Tax
antibody, Tax-specific CTLs, and HTLV-1 proviral load,
and discuss the risk factors for the development of adult
T-cell leukemia/lymphoma and HAM/TSP.

MATERIALS AND METHODS

Subjects and PBMCs

The study population consisted of 45 asymptomatic
carriers who visited Kagoshima University Hospital for
examination to determine HTLV-1 carrier state and for
a clinical check-up during the period from September
2004 to May 2005. None of the subjects had
clinical signs, symptoms, or HTLV-1-associated dis-
eases. Informed consent was obtained from all subjects
and the study protocol was reviewed and approved by
the Medical Ethical Committee of Kagoshima Univer-
sity. Blood samples obtained at clinical visits were
collected in EDTA tubes for determination of the
complete blood counts and differential counts using
standard methods. Ably were counted by a hematologist
during microscopic review of peripheral blood smears,
which were fixed in methanol and stained with Giemsa.
Ably were identified according to the criteria reported
previously [Kondo et al., 1985]. The number of Ably
among 100 leukocytes was recorded as a percentage.
PBMCs were separated from heparinized whole blood by
centrifugation on Ficoll/Hypaque (Pharmacia, Uppsala,
Sweden). For subsequent experiments, the cells were
cryopreserved in liquid nitrogen until assayed as
described previously [Katahira et al., 1995].

HLA Typing of PBMCs

In our recent study, analysis of HLA type revealed
that more than 80% of asymptomatic HTLV-1 carriers
and patients with adult T-cell leukemia/lymphoma
had HLA-A*02 or HLA-A*24 [Kozako et al., 2006],
consistent with other reports of HLA allele types in the
population of Southern Kyushu [Sonoda, 2003]. HLA
types were determined by serological tests using mono-
clonal antibodies (mAbs) for HLA-A*02 (clone: BB7.2)
and HLA-A*24 (clone: 17A10; kindly provided by
Medical and Biological Laboratories, Nagoya, Japan).
Indirect staining was completed by incubation of cells
with goat anti-mouse IgG-fluorescein isothiocyanate
(FITC) (Immunotech, Miami, FL). HLA allele types
were also confirmed by the Luminex method using DNA
isolated from the cryopreserved PBMCs as described (G
& G Science, Fukushima, Japan) [Kikkawa et al., 2003].

Tetramer Assay for HTLV-1-Specific CTL

We used 14 distinct phycoerythrin (PE)-conjugated
HLA-A*0201 and HLA-A*2402 tetramers for HTLV-1

Tax and Env peptides (Medical and Biological Laboratories) in reference to the previous HTLV-1 Tax and Env CTL epitope mapping data [Yashiki et al., 2001]. In addition, two HTLV-1/HLA tetramers were purchased from Beckman Coulter (Fullerton, CA). Finally, 16 HTLV-1/HLA tetramers were prepared for the present study. The 16 epitopes were as follows: Tax11-19 (LLFGYPVYV), Tax123-131 (TLGQHLPTL), Tax155-163 (YLYQLSPPI), Tax178-186 (QLGAFLTNV), Tax307-315 (LLFEEYTNI), Env175-183 (FLNTEPSQL), Env239-247 (VLYSPNVSV), and Env442-450 (ALQTGITLV) for HLA-A*0201, Tax12-20 (LFGYPVYVF), Tax187-195 (PYKRIEELL), Tax289-297 (SFLLSHGLI), Tax301-309 (SFHSLHLLF), Tax311-319 (EYTNIPISL), Env11-19 (FFQFCPLIF), Env21-29 (DYSPSCCTL), and Env153-161 (HFSKCGFPF) for HLA-A*2402 (anchor motifs are indicated in boldface). The quality of each HLA-tetramer was tested by HPLC and biological assay for respective CTLs. Aliquots of 1×10^6 freshly isolated PBMCs were reacted with the 16 HLA tetramers for 45 min at 4°C with FITC-conjugated mouse anti-human CD8 mAbs according to the manufacturer's instructions (Medical and Biological Laboratories). All samples were also examined for surface markers using mouse anti-CD4-PE (Beckman Coulter) and anti-CD45-Peridinin Chlorophyll-a Protein (Becton Dickinson, San Jose, CA). Aliquots of 1×10^5 CD45-positive lymphocytes in fresh samples were analyzed on a FACScan using CellQuest software (Becton Dickinson) [Bieganowska et al., 1999; Kuzushima et al., 2001].

Detection of Anti-HTLV-1 and Anti-Tax Antibody

Anti-HTLV-1 antibody titer and anti-Tax antibody reactivity were examined in the 45 subjects. Anti-HTLV-1 antibody was measured by electrochemiluminescence immunoassay (ECLIA) (Picolumi®HTLV-I; Eisai, Tokyo, Japan) in our hospital, using beads coated with purified HTLV-1 antigen and synthetic Env peptides. Anti-Tax antibody was measured by ELISA, using a recombinant Tax protein expressed in *Escherichia coli* with a full-length HTLV-1 tax gene. The cut-off value was determined from the average of the ELISA absorbance value plus three SD obtained from 169 specimens negative for anti-Tax antibody (Eisai) [Kamihira et al., 1989; Sawada et al., 1989; Kashiwagi et al., 1990]. The levels of antibodies were estimated using a cut-off index (CI): the corresponding serum was considered ELISA-positive at CI ≥ 1.1.

Real-Time PCR Quantitation of HTLV-1 Proviral Load in PBMCs

The standard HTLV-1 DNA was prepared from MT-2 cells [Miyoshi et al., 1981]. Real-time PCR quantitation of HTLV-1 DNA was performed in a Light-Cycler System (Roche Diagnostics, Mannheim, Germany) using measurements of 12 test samples with standard DNA for each assay. The HTLV-1 primer set corresponded to the highly conserved HTLV-1 pX region, SK43, and SK44 [Ehrlich et al., 1990]. The HTLV-1 pX probe set was designed for the two adjacent parts of the pX region, which were labeled with different fluorophores [Sonoda et al., 2004].

The HTLV-1 proviral load was expressed as number of copies per 1,000 cells using the following formula: HTLV-1 proviral load = [(HTLV-1 pX copy number)/ (β-globin copy number/2)] × 1,000. The limit of detection of this method was 0.2 copies of HTLV-1 provirus/ 1,000 cells. Real-time PCR quantitation of HTLV-1 provirus DNA has an inherent error of 25% in terms of coefficient of variation (CV), as seen in different inter-assay runs for HTLV-1 proviral load [Nagai et al., 1998]. However, the present real-time PCR quantitation with duplicate intra-assay reduced the CV to less than 8% [Sonoda et al., 2004]. Using this method, we could perform accurate measurements of HTLV-1 proviral load in PBMCs of asymptomatic HTLV-1 carriers.

Statistical Analysis

Correlation analyses were performed based on either Pearson or Spearman's rho correlation coefficients as appropriate. The resulting values for the two subject groups were compared with Mann–Whitney U tests. SPSS for Windows (version 14.0J; SPSS, Inc., Chicago, IL) was used for statistical analysis, and $P < 0.05$ was considered significant.

RESULTS

Characteristics of the Subjects

The age, sex, and HTLV-1-related markers of these 45 asymptomatic carriers (18 men and 27 women) are summarized in Table I. The subjects' median age was 58 years old with a range from 22 to 82. The median leukocyte count was 5,200/μl with a range from 1,900 to 9,500, and the median Ably level was 1% with a range from 0 to 6% (data not shown). In the present study, overt adult T-cell leukemia/lymphoma, including smoldering type, was excluded by Southern blot hybridization of HTLV-1 proviral DNA. The two cases with Ably levels of more than 5% in the present study (case #12, 6%; case #36, 5%) were also diagnosed as carriers, as no band was detected by Southern blot hybridization.

All 45 cases were positive for anti-HTLV-1 antibodies, and the titers ranged from 2.8 to 330.9 CI with a median value of 81.6. On the other hand, anti-Tax antibody was found in 15 of the 45 cases (33%). One subject (case #43) could not be included in the HTLV-1 proviral load analysis, as his PBMCs were no longer available. The median proviral load was 62.3/1,000 copies ranging from 4.6 to 592.4.

Ten of the 45 carriers had HLA-A*0201, 25 had HLA-A*2402, 8 had both alleles in heterozygous combination, and 2 had neither HLA-A*0201 nor HLA-A*2402. As the HLA-tetramers in the present study can detect HTLV-1-specific CD8+ cells possessing either HLA-A*0201 or HLA-A*2402, 43 cases were examined for

TABLE I. HTLV-1-Related Markers Among the 45 Asymptomatic Carriers

Case No.	Age	Sex	Anti-HTLV-1 antibody titer (CI)	Anti-Tax antibody titer (CI)	Anti-Tax antibody positivity	Tax11-19-specific CTL (%)	Tax301-309-specific CTL (%)	Proviral load (/1,000 copies)
HLA-A*0201-related								
1	72	F	5.5	0.21	−	0.03	NA	21.0
2	74	M	23.71	0.24	−	0.00	NA	12.2
3	46	F	6.1	0.21	−	0.08	NA	24.1
4	53	M	41.8	1.79	+	0.29	NA	39.7
5	47	F	16.8	0.68	−	0.00	NA	71.8
6	56	F	141.5	0.29	−	0.00	NA	17.3
7	54	M	30.92	7.25	+	0.09	NA	63.2
8	66	F	2.9	0.26	−	0.00	NA	25.1
9	51	F	225.6	0.61	−	0.00	NA	128.7
10	62	F	20.46	0.82	−	0.01	NA	9.8
HLA-A*0201 and HLA-A*2402-related								
11	80	F	20.0	1.71	+	0.73	0.07	42.7
12	63	M	282.4	0.71	−	0.02	0.23	469.3
13	70	F	89.47	0.26	−	0.03	0.02	135.4
14	47	F	30.1	0.29	−	0.00	0.00	12.2
15	61	F	37.8	0.29	−	0.04	0.00	5.2
16	72	F	23.5	0.40	−	0.00	0.00	22.4
17	62	F	2.8	1.24	+	0.14	0.04	4.6
18	53	M	145.7	1.61	+	0.03	0.01	4.8
HLA-A*2402-related								
19	58	F	256.4	1.76	+	NA	0.16	106.9
20	52	M	81.6	0.29	−	NA	0.50	61.4
21	47	M	142.8	0.24	−	NA	0.14	69.5
22	51	F	70.8	0.29	−	NA	0.00	338.3
23	22	F	198.0	2.71	+	NA	1.11	93.7
24	68	F	101.5	0.35	−	NA	0.00	79.8
25	69	F	241.5	0.29	−	NA	0.00	271.2
26	80	F	288.8	6.82	+	NA	0.55	82.3
27	53	F	117.1	0.35	−	NA	0.06	592.4
28	58	F	101.2	0.32	−	NA	0.15	107.3
29	82	M	39.85	0.74	−	NA	0.12	26.1
30	68	F	76.46	5.85	+	NA	0.00	27.3
31	66	F	12.25	0.21	−	NA	0.31	54.6
32	68	M	147.39	0.50	−	NA	0.17	213.4
33	31	M	159.4	0.29	−	NA	0.47	69.5
34	73	M	62.8	0.48	−	NA	0.15	77.9
35	55	F	139.71	6.75	+	NA	1.05	73.2
36	58	M	231.7	2.14	+	NA	1.37	27.0
37	57	M	11.1	2.25	+	NA	0.19	11.4
38	70	F	330.9	5.01	+	NA	3.25	40.4
39	44	M	46.0	0.72	−	NA	0.01	144.5
40	78	M	73.6	0.54	−	NA	0.19	50.9
41	61	M	95.4	2.43	+	NA	0.16	68.4
42	76	F	169.42	1.52	+	NA	0.41	41.0
43	58	F	76.34	0.91	−	NA	0.60	NA
Irrelevant HLA alleles								
44	43	M	121.2	0.18	−	NA	NA	97.1
45	53	M	162.5	0.26	−	NA	NA	265.9

NA, not available.

each tetramer-CD8$^+$ cells. The cases with more than 0.03% tetramer-CD8$^+$ cells distributed as a cluster were judged as positive for HTLV-1-specific CD8$^+$ cells in the present study. Most subjects had single epitope-specific CTLs for each HLA allele: Tax11-19-specific CTL for HLA-A*0201 and Tax301-309-specific CTL for HLA-A*2402. A few subjects had multiple epitope-specific CTLs for one HLA allele, including the major epitope-specific CTLs mentioned above and a few minor epitope-specific CTLs. In the present study, we assessed only the major CTLs in both HLA alleles, as shown in Table I.

Nine of the 18 (50%) cases with HLA-A*0201 were positive for Tax11-19-specific CTLs, and 23 of the 33 (69.7%) cases with HLA-A*2402 were positive for Tax301-309-specific CTLs. Only two cases (case #16: 0.08% and case #35: 0.06%) had sufficient Env-specific CTLs.

Relationships Between HTLV-1-Related Markers

Anti-HTLV-1 antibody titer in the anti-Tax antibody-positive group was not different from that in the

antibody-negative group (median 139.7 vs. 75.0, P = 0.194, Mann–Whitney; Fig. 1). There was no significant correlation between anti-HTLV-1 antibody titer and the frequency of Tax11-19-specific CTLs (P = 0.628, Spearman; Fig. 2A), whereas the frequency of Tax11-19-specific CTLs was significantly higher in the anti-Tax antibody-positive group compared with the antibody-negative group among HLA-A*0201-positive cases (median 0.14 vs. 0.00%, P = 0.002, Mann–Whitney; Fig. 2B). On the other hand, there was a significant positive correlation between anti-HTLV-1 antibody titer and the frequency of Tax301-309-specific CTLs (r = 0.549, P = 0.001, Pearson; Fig. 2C), and the frequency of Tax301-309-specific CTLs was also significantly higher in the anti-Tax antibody-positive group compared with the antibody-negative group among HLA-A*2402-positive cases (median 0.19 vs. 0.09%, P = 0.033, Mann–Whitney; Fig. 2D).

There was a significant positive correlation between anti-HTLV-1 antibody titer and HTLV-1 proviral load (r = 0.353, P = 0.019, Pearson; Fig. 3A). However, the proviral load in the anti-Tax antibody-positive group was not different from and even seemed slightly lower than that in the antibody-negative group (median 41.0 vs. 69.5/1,000 copies, P = 0.147, Mann–Whitney; Fig. 3B). Next, we analyzed the relationship between Tax-specific CTLs and proviral load. As shown in Figure 4, the frequency of Tax11-19-specific CTLs was significantly lower than that of Tax301-309-specific CTLs (the frequencies of Tax301-309-specific CTLs in Tax11-19-specific CTL-positive carriers were excluded previously) in the CTL-positive carriers (median 0.08 vs. 0.19%, P = 0.009, Mann–Whitney). However, the proviral load in the Tax11-19-specific CTL-positive group was significantly lower than that in the Tax11-19-specific CTL-negative group consisting of cases with

Tax301-309-specific CTL alone or neither CTL (median 24.1 vs. 69.5/1,000 copies, P = 0.017, Mann–Whitney; Fig. 5A). On the other hand, the proviral load in the Tax301-309-specific CTL-positive group was not different from that in the CTL-negative group consisting of cases with Tax11-19-specific CTL alone or neither CTL (median 69.0 vs. 33.5/1,000 copies, P = 0.291, Mann–Whitney; Fig. 5B). The reason that the Tax301-309-specific CTL-positive group did not have lower proviral load than the CTL-negative group can be explained by the inclusion in the Tax301-309-specific CTL-negative group of many cases with Tax11-19-specific CTLs as very strong negative regulators of HTLV-1 proviral load (7 of 22 carriers) [Jeffery et al., 1999, 2000; Nagai et al., 2001; Vine et al., 2002; Yao et al., 2006]. Therefore, we next analyzed the relationship between Tax301-309-specific CTLs and proviral load among cases positive for HLA-A*2402 but not A*0201. These subjects were divided into a higher Tax301-309-specific CTL frequency group (>median: 0.17%, n = 11; case #43 was not included as mentioned above) and a lower CTL frequency group (≤0.17%, n = 13), and the proviral load in the former was significantly lower than that in the latter (median 54.6 vs. 106.9/1,000 copies, P = 0.015, Mann–Whitney; Fig. 6).

DISCUSSION

The present study was performed to examine the relationships between anti-HTLV-1 antibody, anti-Tax antibody, Tax-specific CTLs, and HTLV-1 proviral load in asymptomatic carriers. Anti-HTLV-1 antibody is predominantly directed against structural polypeptides of HTLV-1, such as Env or Gag, but rarely against Tax [Yamamoto et al., 1983; Schneider et al., 1984]. Therefore, it is likely that anti-HTLV-1 and anti-Tax antibodies are each controlled by independent regulation of production. We found no significant correlation between anti-Tax antibody positivity and anti-HTLV-1 antibody titer in the present study (Fig. 1). On the other hand, the frequency of Tax11-19-specific CTLs showed a significant positive correlation only with anti-Tax antibody positivity, but not with anti-HTLV-1 antibody titer, although the frequency of Tax301-309-specific CTLs showed significant positive correlations with both types of antibody (Fig. 2). These findings imply that anti-Tax antibody is associated with Tax-specific CTLs, and suggest a strong positive correlation between humoral and cellular immunity against HTLV-1 Tax. A recent study demonstrated that long-lasting CD4+ T-cell memory depends on the presence of B cells retaining antigen [van Essen et al., 2000]. Furthermore, polyclonal activation of B cells may help to optimize the memory CTL response against persistent viruses that are predominantly controlled by CTL [Matter et al., 2005]. These mechanisms may help explain the close relationship between the anti-Tax antibody and the frequency of Tax-specific CTLs that we report here. Perhaps anti-Tax antibody can serve as a convenient marker for the presence of Tax-specific CTLs.
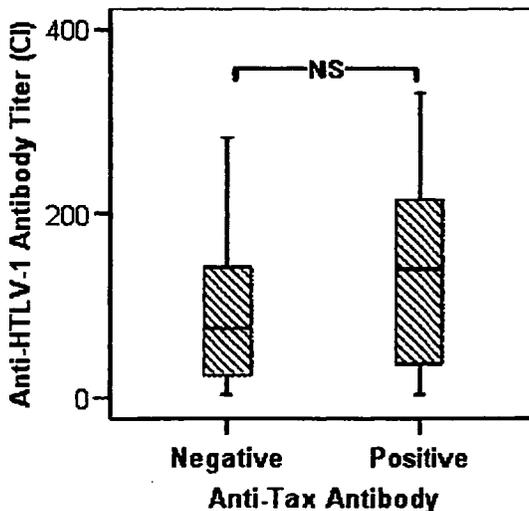

Fig. 1. Comparison of anti-HTLV-1 antibody titer between the anti-Tax antibody-positive and negative groups. Anti-HTLV-1 antibody titer in the anti-Tax antibody-positive group was not different from that in the antibody-negative group (median 139.7 vs. 75.0, P = 0.194, Mann–Whitney).
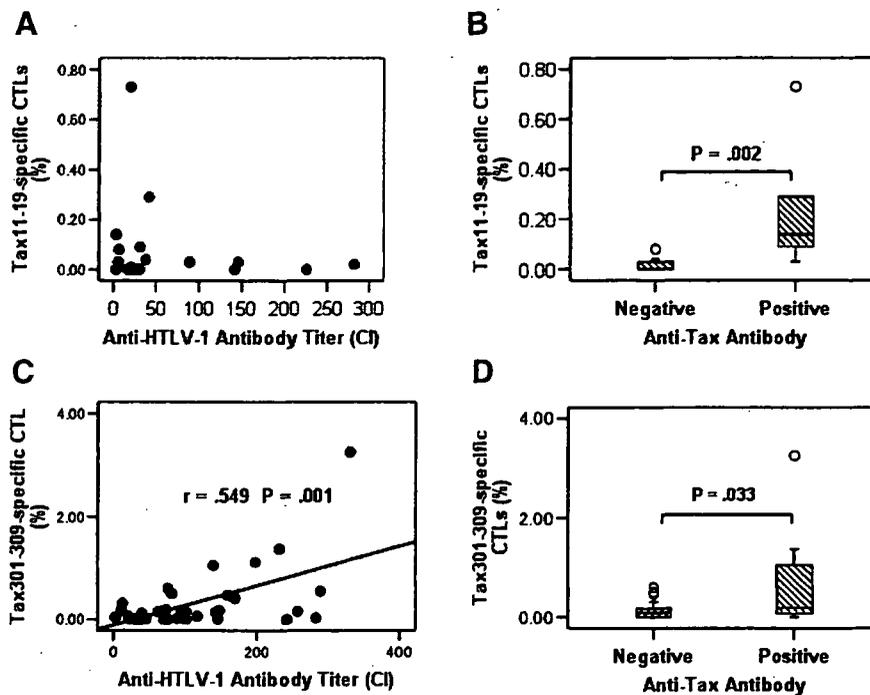
A

B

C

D

Fig. 2. . Relationships between the frequencies of Tax-specific CTLs and anti-HTLV-1 or anti-Tax antibody. There was no significant correlation between anti-HTLV-1 antibody titer and the frequency of Tax11-19-specific CTLs ($P = 0.628$, Spearman) (A), whereas the frequency of Tax11-19-specific CTLs was significantly higher in the anti-Tax antibodypositive group compared with the antibody-negative group among HLA-A*0201-positive cases (median 0.14 vs. 0.00%,

$P = 0.002$, Mann–Whitney) (B). There was a significant positive correlation between anti-HTLV-1 antibody titer and the frequency of Tax301-309-specific CTLs ($r = 0.549$, $P = 0.001$, Pearson) (C), and the frequency of Tax301-309-specific CTLs was also significantly higher in the anti-Tax antibody-positive group compared with the antibody-negative group among HLA-A*2402-positive cases (median 0.19 vs. 0.09%, $P = 0.033$, Mann–Whitney) (D).

As predicted from the findings of the Miyazaki Cohort Study [Ishihara et al., 1994], we found a strong positive correlation between anti-HTLV-1 antibody and HTLV-1 proviral load among asymptomatic carriers. However, it remains unclear whether this high antibody titer contributes significantly to controlling the equilibrium proviral load. We observed very few Env-specific CTLs in asymptomatic carriers which further suggests insufficient collaboration of humoral and cellular immunity against Env protein. The positive relationship between anti-HTLV-1 antibody and proviral load can be explained by induction of high anti-HTLV-1 antibody titer in response to a high proviral load. In the absence of sufficient virus-specific CTLs, however, the proviral load remains elevated. In contrast, a tendency toward a negative relationship between the anti-Tax antibody positivity and the proviral load is hinted at in our studies, although statistical analysis did not show a significant correlation between these factors (Fig. 3).

The observed relationship between anti-Tax antibody titer and the proviral load can be explained by presence of high frequency of CTLs against HTLV-1 Tax in anti-Tax antibody-positive carriers. Tax11-19-specific CTLs showed a strong negative relationship to the proviral load (Fig. 5A), and Tax301-309-specific CTLs also showed a negative relationship to that after exclusion of the bias due to Tax11-19-specific CTLs (Fig. 6). As carriers with higher anti-Tax antibody titer will have a higher frequency of Tax-specific CTLs, as demonstrated

above, anti-Tax antibody may play a role in reducing proviral load indirectly, although it will not kill the virus-infected cells directly.

Previous studies have strongly suggested that elevated proviral loads are closely related with the development of adult T-cell leukemia/lymphoma [Kinoshita et al., 1985; Yokota et al., 1989; Manns et al., 1999; Taylor et al., 1999; Okayama and Stuver, 2003]. Our findings also indicate that HTLV-1 carriers from the Miyazaki Cohort Study with the highest anti-HTLV-1 antibody titers and lowest anti-Tax antibody reactivity may be at greatest risk for adult T-cell leukemia/lymphoma.

On the other hand, previous studies have suggested that CTLs may be useful to determine the proviral load and the risk of proinflammatory disease [Bangham et al., 1996, 1999]. It is possible that HTLV-1-specific CTLs exert both protective and inflammatory effects. There is also evidence that HTLV-1-specific CTL may contribute to the inflammation seen in HAM/TSP [Elovaara et al., 1993; Ijichi et al., 1993; Biddison et al., 1997; Levin et al., 1997; Kubota et al., 1998]. However, frequent and chronically activated HTLV-1-specific CTLs have been found in healthy carriers as well as in HAM/TSP patients [Parker et al., 1992, 1994; Daenke et al., 1996; Jeffery et al., 1999]. Therefore, previous studies suggested that the CTLs efficiently destroy HTLV-1-infected cells *in vivo* and thus protect against inflammatory diseases, such as HAM/TSP
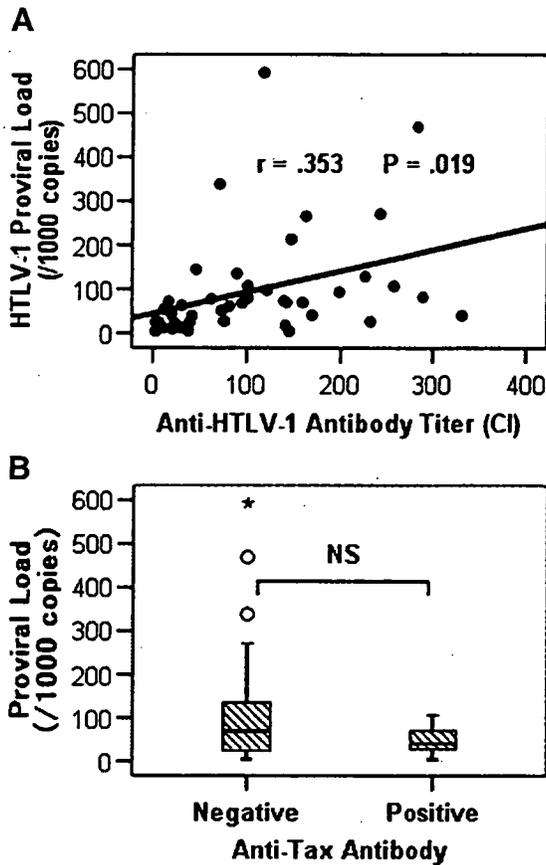
A

B

Fig. 3. Relationships between HTLV-1 proviral load and anti-HTLV-1 or anti-Tax antibody. There was a significant positive correlation between anti-HTLV-1 antibody titer and HTLV-1 proviral load ($r = 0.353$, $P = 0.019$, Pearson) (A). However, the proviral load in the anti-Tax antibody-positive group was not different from and seemed slightly lower than that in the antibody-negative group (median 41.0 vs. 69.5/1,000 copies, $P = 0.147$, Mann–Whitney) (B).
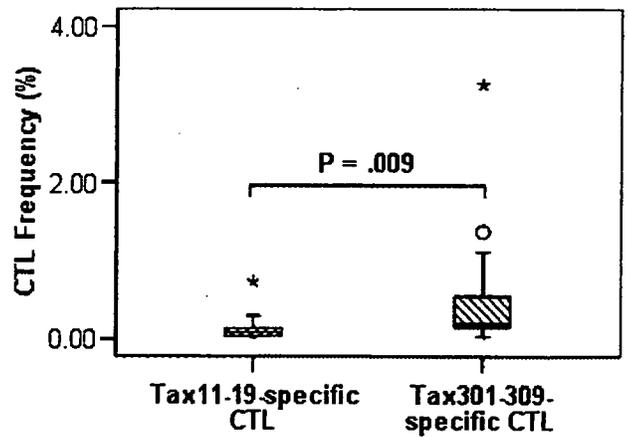

Fig. 4. Comparison of the frequencies between Tax11-19 and Tax301-309-specific CTLs. The frequency of Tax11-19-specific CTLs was significantly lower than that of Tax301-309-specific CTLs in the CTL-positive carriers (median 0.08 vs. 0.19%, $P = 0.009$, Mann–Whitney). The frequencies of Tax301-309-specific CTLs in Tax11-19-specific CTL-positive carriers were excluded previously.
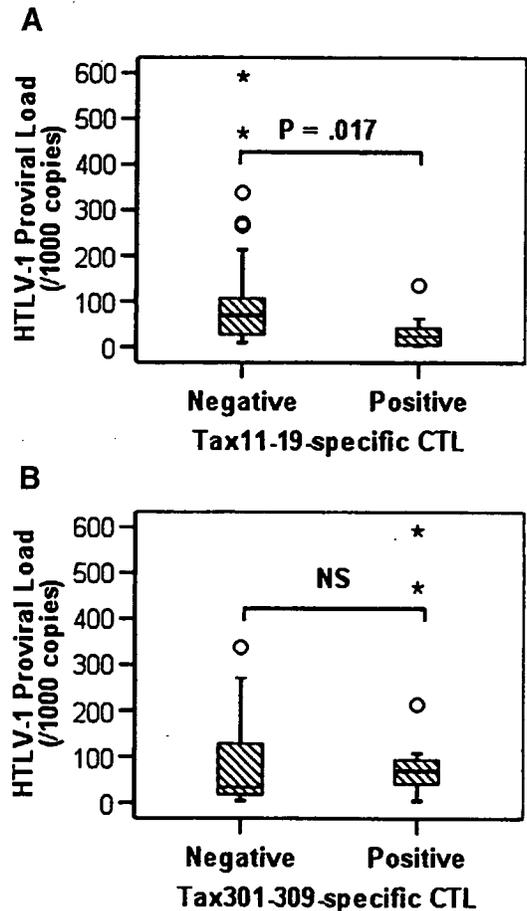
A

B

Fig. 5. Comparison of HTLV-1 proviral load between Tax-specific CTL-positive and CTLnegative groups. HTLV-1 proviral load was significantly lower in the Tax11-19-specific CTL-positive group compared with the CTL-negative group among all cases (median 24.1 vs. 69.5, $P = 0.017$, Mann–Whitney) (A). On the other hand, proviral load was not different between Tax301-309-specific CTL-positive and CTL-negative groups (median 69.0 vs. 33.5/1,000 copies, $P = 0.291$, Mann–Whitney) (B).

[Niewiesk et al., 1994; Hanon et al., 2000]. In addition, a recent case-control study showed that the MHC class I genes HLA-A*02 and/or Cw*08 conferred protection against HAM/TSP; possession of the A*02 and/or Cw*08 genes prevented 36% of potential HAM/TSP cases [Jeffery et al., 1999, 2000]. These observations suggested that both A*02 and Cw*08-restricted CTLs are particularly efficient at recognizing Tax, and reduce the proviral load and hence the risk of disease. In our study, Tax11-19-specific CTLs showed a negative relationship to the proviral load more strongly than Tax301-309-specific CTLs (Fig. 5). This result favors the conclusion of the previous study that demonstrated a protective effect of HLA-A*02 against HTLV-1 proviral load and the risk of HAM/TSP in virus-infected individuals, as the Tax11-19-specific CTLs are HLA-A*0201-restricted [Jeffery et al., 1999]. Tax11-19-specific CTLs will proliferate rapidly in response to HTLV-1 Tax, and kill HTLV-1-infected cells rapidly, and so limit the proviral load to a low level. However, the proliferation rate of the CTLs will not be stimulated to a greater extent by the proviral load when a strong immune response reaches equilibrium with a low
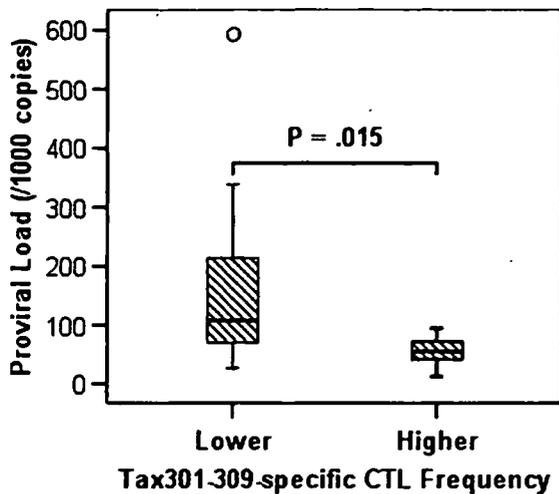
Fig. 6. Comparison of HTLV-1 proviral load between groups with higher and lower frequencies of Tax301-309-specific CTLs. HTLV-1 proviral load was significantly lower in the group with higher frequency of Tax301-309-specific CTLs (>0.17%) compared with the group with lower frequency of these CTLs (≤0.17%) (median 54.6 vs. 106.9/1,000 copies, $P = 0.015$, Mann–Whitney) among cases positive for HLA-A*2402 but not A*0201.

proviral load. Therefore, previous reports have focused on the 'efficiency' of the CTL response as a factor of reduction in the proviral load rather than the frequency of specific CTLs, which was considered an unreliable index of the effectiveness of the CTL response [Bangham, 2003]. In fact, both experiment [Ogg et al., 1998; Kubota et al., 2000; Wodarz and Bangham, 2000; Betts et al., 2001; Wodarz et al., 2001] and theory [Bangham, 2002; Addo et al., 2003] have shown various results indicating positive, negative, or zero correlations between the specific CTL frequency and virus load. In support of this idea, the frequency of Tax11-19-specific CTLs was significantly lower than that of Tax301-309-specific CTLs in the present study (Fig. 4). However, the higher frequency of Tax301-309-specific CTLs also showed a negative relationship to the proviral load after exclusion of the bias due to Tax11-19-specific CTLs (Fig. 6). As Tax301-309-specific CTLs may make a weaker CTL response to HTLV-1 than Tax11-19-specific CTLs, these CTLs may have to proliferate more frequently to reduce the proviral load sufficiently. The results of the present study demonstrate that a high frequency of Tax-specific CTLs can maintain a relatively low HTLV-1 proviral load and can be a factor in reduction of the proviral load in healthy carriers in addition to 'CTL efficiency'.

However, the present study had the limitation that our method can detect only HTLV-1-specific CD8+ cells possessing either HLA-A*0201 or A*2402. Although more than 80% of HTLV-1 carriers in the population of Southern Kyushu had HLA-A*02 or A*24, other HLA class I haplotype-restricted CTLs should be analyzed to gain a greater understanding of the whole immunity against HTLV-1 Tax.

In conclusion, we report a correlation between anti-Tax antibody and the frequency of Tax-specific CTLs.

Anti-Tax antibody and Tax-specific CTLs may prevent growth of HTLV-1-infected cells in carriers. In addition, higher anti-HTLV-1 antibody titer is associated with a higher HTLV-1 proviral load. Further studies are required to focus on how HTLV-1-related markers and the host immune response impact on the development of adult T-cell leukemia/lymphoma and HAM/TSP.

ACKNOWLEDGMENTS

REFERENCES

Addo MM, Yu XG, Rathod A, Cohen D, Eldridge RL, Strick D, Johnston MN, Corcoran C, Wurcel AG, Fitzpatrick CA, Feeney ME, Rodriguez WR, Basgoz N, Draenert R, Stone DR, Brander C, Goulder PJ, Rosenberg ES, Altfeld M, Walker BD. 2003. Comprehensive epitope analysis of human immunodeficiency virus type 1 (HIV-1)-specific T-cell responses directed against the entire expressed HIV-1 genome demonstrate broadly directed responses, but no correlation to viral load. J Virol 77:2081–2092.

Bangham CRM. 2002. Genetics and dynamics of the immune response to HTLV-I. Gann Monogr Cancer Res 50:397–402.

Bangham CRM. 2003. The immune control and cell-to-cell spread of human T-lymphotropic virus type 1. J Gen Virol 84:3177–3189.

Bangham C, Kermode A, Hall S, Daenke S. 1996. The cytotoxic T lymphocyte response to HTLV-I: The main determinant of disease? Semin Virol 7:41.

Bangham CR, Hall SE, Jeffery KJ, Vine AM, Witkover A, Nowak MA, Wodarz D, Usuku K, Osame M. 1999. Genetic control and dynamics of the cellular immune response to the human T-cell leukaemia virus, HTLV-I. Philos Trans R Soc Lond B Biol Sci 354:691–700.

Betts MR, Ambrozak DR, Douek DC, Bonhoeffer S, Brenchley JM, Casazza JP, Koup RA, Picker LJ. 2001. Analysis of total human immunodeficiency virus (HIV)-specific CD4+ and CD8+ T-cell responses: Relationship to viral load in untreated HIV infection. J Virol 75:11983–11991.

Biddison WE, Kubota R, Kawanishi T, Taub DD, Cruikshank WW, Center DM, Connor EW, Utz U, Jacobson S. 1997. Human T cell leukemia virus type I (HTLV-I)-specific CD8+ CTL clones from patients with HTLV-I-associated neurologic disease secrete proinflammatory cytokines, chemokines, and matrix metalloproteinase. J Immunol 159:2018–2025.

Bieganowska K, Hollsberg P, Buckle GJ, Lim DG, Greten TF, Schneck J, Altman JD, Jacobson S, Ledis SL, Hanchard B, Chin J, Morgan O, Roth PA, Hafler DA. 1999. Direct analysis of viral-specific CD8+ T cells with soluble HLA-A2/Tax 11-19 tetramer complexes in patients with human T-lymphotropic virus-associated myelopathy. J Immunol 162:1765–1771.

Daenke S, Kermode AG, Hall SE, Taylor G, Weber J, Nightingale S, Bangham CR. 1996. High activated and memory cytotoxic T-cell responses to HTLV-1 in healthy carriers and patients with tropical spastic paraparesis. Virology 217:139–146.

Ehrlich G, Greenberg S, Abbot M. 1990. Detection of human T-cell lymphoma/leukemia viruses. In: Innis M, Gelfand D, Suinsky J, White T, editors. PCR protocols. San Diego: Academic Press. pp 325–332.

Elovaara I, Koenig S, Brewah AY, Woods RM, Lehky T, Jacobson S. 1993. High human T cell lymphotropic virus type 1 (HTLV-1)-specific precursor cytotoxic T lymphocyte frequencies in patients with HTLV-1-associated neurological disease. J Exp Med 177:1567–1573.

Etoh K, Tamiya S, Yamaguchi K, Okayama A, Tsubouchi H, Ideta T, Mueller N, Takatsuki K, Matsuoka M. 1997. Persistent clonal

proliferation of human T-lymphotropic virus type I-infected cells in vivo. Cancer Res 57:4862–4867.

Gessain A, Barin F, Vernant JC, Gout O, Maurs L, Calender A, de The G. 1985. Antibodies to human T-lymphotropic virus type-I in patients with tropical spastic paraparesis. Lancet 2:407–410.

Hanon E, Hall S, Taylor GP, Saito M, Davis R, Tanaka Y, Usuku K, Osame M, Weber JN, Bangham CR. 2000. Abundant tax protein expression in CD4+ T cells infected with human T-cell lymphotropic virus type I (HTLV-I) is prevented by cytotoxic T lymphocytes. Blood 95:1386–1392.

Hino S, Yamaguchi K, Katamine S, Sugiyama H, Amagasaki T, Kinoshita K, Yoshida Y, Doi H, Tsuji Y, Miyamoto T. 1985. Mother-to-child transmission of human T-cell leukemia virus type-I. Jpn J Cancer Res 76:474–480.

Hinuma Y, Nagata K, Hanaoka M, Nakai M, Matsumoto T, Kinoshita KI, Shirakawa S, Miyoshi I. 1981. Adult T-cell leukemia: Antigen in an ATL cell line and detection of antibodies to the antigen in human sera. Proc Natl Acad Sci USA 78:6476–6480.

Hisada M, Okayama A, Tachibana N, Stuver SO, Spiegelman DL, Tsubouchi H, Mueller NE. 1998a. Predictors of level of circulating abnormal lymphocytes among human T-lymphotropic virus type I carriers in Japan. Int J Cancer 77:188–192.

Hisada M, Okayama A, Shioiri S, Spiegelman DL, Stuver SO, Mueller NE. 1998b. Risk factors for adult T-cell leukemia among carriers of human T-lymphotropic virus type I. Blood 92:3557–3561.

Hisada M, Okayama A, Spiegelman D, Mueller NE, Stuver SO. 2001. Sex-specific mortality from adult T-cell leukemia among carriers of human T-lymphotropic virus type I. Int J Cancer 91:497–499.

Ijichi S, Izumo S, Eiraku N, Machigashira R, Kubota M, Nagai M, Ikegami N, Kashio N, Umehara I, Maruyama I, Osame M. 1993. An autoaggressive process against bystander tissues in HTLV-I-infected individuals: A possible pathomechanism of HAM/TSP. Med Hypotheses 41:542–547.

Ishihara S, Okayama A, Stuver S, Horinouchi H, Shioiri S, Murai K, Kubota T, Yamashita R, Tachibana N, Tsubouchi H, Mueller N. 1994. Association of HTLV-I antibody profile of asymptomatic carriers with proviral DNA levels of peripheral blood mononuclear cells. J Acquir Immune Defic Syndr 7:199–203.

Jacobson S, Shida H, McFarlin DE, Fauci AS, Koenig S. 1990. Circulating CD8+ cytotoxic T lymphocytes specific for HTLV-I pX in patients with HTLV-I associated neurological disease. Nature 348:245–248.

Jeffery KJ, Usuku K, Hall SE, Matsumoto W, Taylor GP, Procter J, Bunce M, Ogg GS, Welsh KI, Weber JN, Lloyd AL, Nowak MA, Nagai M, Kodama D, Izumo S, Osame M, Bangham CR. 1999. HLA alleles determine human T-lymphotropic virus-I (HTLV-I) provirus load and the risk of HTLV-I-associated myelopathy. Proc Natl Acad Sci USA 96:3848–3853.

Jeffery KJ, Siddiqui AA, Bunce M, Lloyd AL, Vine AM, Witkover AD, Izumo S, Usuku K, Welsh KI, Osame M, Bangham CR. 2000. The influence of HLA class I alleles and heterozygosity on the outcome of human T cell lymphotropic virus type I infection. J Immunol 165:7278–7284.

Kamihira S, Toriya K, Amagasaki T, Momita S, Ikeda S, Yamada Y, Tomonaga M, Ichimaru M, Kinoshita K, Sawada T. 1989. Antibodies against p40tax gene product of human T-lymphotropic virus type-I (HTLV-I) under various conditions of HTLV-I infection. Jpn J Cancer Res 80:1066–1071.

Kannagi M, Sugamura K, Sato H, Okochi K, Uchino H, Hinuma Y. 1983. Establishment of human cytotoxic T cell lines specific for human adult T cell leukemia virus-bearing cells. J Immunol 130:2942–2946.

Kannagi M, Sugamura K, Kinoshita K, Uchino H, Hinuma Y. 1984. Specific cytolysis of fresh tumor cells by an autologous killer T cell line derived from an adult T cell leukemia/lymphoma patient. J Immunol 133:1037–1041.

Kannagi M, Harada S, Maruyama I, Inoko H, Igarashi H, Kuwashima G, Sato S, Morita M, Kidokoro M, Sugimoto M, Funahashi S, Osame M, Shida H, Honjo T. 1991. Predominant recognition of human T cell leukemia virus type I (HTLV-I) pX gene products by human CD8+ cytotoxic T cells directed against HTLV-I-infected cells. Int Immunol 3:761–767.

Kannagi M, Matsushita S, Harada S. 1993. Expression of the target antigen for cytotoxic T lymphocytes on adult T-cell leukemia cells. Int J Cancer 54:582–588.

Kannagi M, Matsushita S, Shida H, Harada S. 1994. Cytotoxic T cell response and expression of the target antigen in HTLV-I infection. Leukemia 8:S54–S59.

Kashiwagi S, Kajiyama W, Hayashi J, Noguchi A, Nakashima K, Nomura H, Ikematsu H, Sawada T, Kida S, Koide A. 1990. Antibody to p40tax protein of human T cell leukemia virus 1 and infectivity. J Infect Dis 161:426–429.

Katahira Y, Yashiki S, Fujiyoshi T, Nomura K, Tara M, Mori M, Setoyama M, Kanzaki T, Shida H, Sonoda S. 1995. In vitro induction of cytotoxic T lymphocytes against HTLV-I-infected T-cells from adult T-cell leukemia patients, asymptomatic HTLV-I carriers and seronegative healthy donors. Jpn J Cancer Res 86:21–27.

Kikkawa E, Miyahara N, Narue TK, Shimada K, Azuma F, Hara H, Inoko H. 2003. Evaluation of the PCR-Luminex method for four-digital level genotyping of the HLA-A, HLA-B and HLA-DRB1 genes in the Japanese population. MHC 10:21–31.

Kinoshita K, Amagasaki T, Ikeda S, Suzuyama J, Toriya K, Nishino K, Tagawa M, Ichimaru M, Kamihira S, Yamada Y. 1985. Preleukemic state of adult T cell leukemia: Abnormal T lymphocytosis induced by human adult T cell leukemia-lymphoma virus. Blood 66:120–127.

Kinoshita K, Amagasaki T, Hino S, Doi H, Yamanouchi K, Ban N, Momita S, Ikeda S, Kamihira S, Ichimaru M. 1987. Milk-borne transmission of HTLV-I from carrier mothers to their children. Jpn J Cancer Res 78:674–680.

Kondo T, Nonaka H, Miyamoto N, Hanaoka M. 1985. Very low percentage flower cell carrier—Can it be an index for pre-leukemic stage for ATLL? Oncologia 12:139.

Kozako T, Arima N, Toji S, Masamoto I, Akimoto M, Hamada H, Che XF, Fujiwara M, Matsushita K, Tokunaga M, Haraguchi K, Uozumi K, Suzuki S, Takezaki T, Sonoda S. 2006. Reduced frequency, diversity, and function of human T cell leukemia virus type 1-specific CD8+ T cell in adult T cell leukemia patients. J Immunol 177:5718–5726.

Kubota R, Kawanishi T, Matsubara H, Manns A, Jacobson S. 1998. Demonstration of human T lymphotropic virus type I (HTLV-I) tax-specific CD8+ lymphocytes directly in peripheral blood of HTLV-I-associated myelopathy/tropical spastic paraparesis patients by intracellular cytokine detection. J Immunol 161:482–488.

Kubota R, Kawanishi T, Matsubara H, Manns A, Jacobson S. 2000. HTLV-I specific IFN-γ+ CD8+ lymphocytes correlate with the provirus load in peripheral blood of infected individuals. J Neuroimmunol 102:208–215.

Kuzushima K, Hayashi N, Kimura H, Tsurumi T. 2001. Efficient identification of HLA-A*2402-restricted cytomegalovirus-specific CD8+ T-cell epitopes by a computer algorithm and an enzyme-linked immunospot assay. Blood 98:1872–1881.

Levin MC, Lehky TJ, Flerlage AN, Katz D, Kingma DW, Jaffe ES, Heiss JD, Patronas N, McFarland HF, Jacobson S. 1997. Immunologic analysis of a spinal cord-biopsy specimen from a patient with human T-cell lymphotropic virus type I-associated neurologic disease. N Engl J Med 336:839.

Maloney EM, Blattner WA. 2003. HTLV-I worldwide patterns and disease associations. Gann Monogr Cancer Res 50:339–361.

Manns A, Miley WJ, Wilks RJ, Morgan OS, Hanchard B, Wharfe G, Cranston B, Maloney E, Wells SL, Blattner WA, Waters D. 1999. Quantitative proviral DNA and antibody levels in the natural history of HTLV-I infection. J Infect Dis 180:1487–1493.

Matter M, Mumprecht S, Pinschewer DD, Pavelic V, Yagita H, Krautwald S, Borst J, Ochsenbein AF. 2005. Virus-induced polyclonal B cell activation improves protective CTL memory via retained CD27 expression on memory CTL. Eur J Immunol 35: 3229–3239.

Miyoshi I, Kubonishi I, Yoshimoto S, Shiraishi Y. 1981. A T-cell line derived from normal human cord leukocytes by co-culturing with human leukemic T-cells. Gann 72:978–981.

Nagai M, Usuku K, Matsumoto W, Kodama D, Takenouchi N, Moritoyo T, Hashiguchi S, Ichinose M, Bangham CRM, Izumo S, Osame M. 1998. Analysis of HTLV-I proviral load in 202 HAM/TSP patients and 243 asymptomatic HTLV-I carriers: High proviral load strongly predisposes to HAM/TSP. J Neurovirol 4:586–593.

Nagai M, Kubota R, Greten TF, Schneck JP, Leist TP, Jacobson S. 2001. Increased activated human T cell lymphotropic virus type I (HTLV-I) Tax11-19-specific memory and effector CD8+ cells in patients with HTLV-I-associated myelopathy/tropical spastic paraparesis: Correlation with HTLV-I provirus load. J Infect Dis 183:197–205.

Niewiesk S, Daenke S, Parker CE, Taylor G, Weber J, Nightingale S, Bangham CR. 1994. The transactivator gene of human T-cell leukemia virus type I is more variable within and between healthy carriers than patients with tropical spastic paraparesis. J Virol 68: 6778–6781.

Ogg GS, Jin X, Bonhoeffer S, Dunbar PR, Nowak MA, Monard S, Segal JP, Cao Y, Rowland-Jones SL, Cerundolo V, Hurley A, Markowitz M, Ho DD, Nixon DF, McMichael AJ. 1998. Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. Science 279:2103–2106.

Okayama A, Stuver SO. 2003. Long-term follow-up of HTLV-I carriers. In: Two decades of adult T-cell leukemia and HTLV-I research. Gann Monogr Cancer Res 50:127–139.

Osame M, Usuku K, Izumo S, Ijichi N, Amitani H, Igata A, Matsumoto M, Tara M. 1986. HTLV-I associated myelopathy, a new clinical entity. Lancet 1:1031–1032.

Parker CE, Daenke S, Nightingale S, Bangham CR. 1992. Activated, HTLV-I-specific cytotoxic T lymphocytes are found in healthy seropositives as well as in patients with tropical spastic paraparesis. Virology 188:628–636.

Parker CE, Nightingale S, Taylor GP, Weber J, Bangham CR. 1994. Circulating anti-Tax cytotoxic T lymphocytes from human T-cell leukemia virus type I-infected people, with and without tropical spastic paraparesis, recognize multiple epitopes simultaneously. J Virol 68:2860–2868.

Poiesz BJ, Ruscetti FW, Gazdar AF, Bunn PA, Minna JD, Gallo RC. 1980. Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. Proc Natl Acad Sci USA 77:7415–7419.

Sawada T, Tohmatsu J, Obara T, Koide A, Kamihira S, Ichimaru M, Kashiwagi S, Kajiyama W, Matsumura N, Kinoshita K, Yano M, Yamaguchi K, Kiyokawa T, Takatsuki K, Taguchi H, Miyoshi I. 1989. High risk of mother-to-child transmission of HTLV-I in p40^tax antibody-positive mothers. Jpn J Cancer Res 80:506–508.

Schneider J, Yamamoto N, Hinuma Y, Hunsmann G. 1984. Sera from adult T-cell leukemia patients react with envelope and core polypeptides of adult T-cell leukemia virus. Virology 15:1–11.

Sonoda S. 2003. Genetic risk of disease development among HTLV-I carriers. Gann Monogr Cancer Res 50:289–301.

Sonoda J, Koriyama C, Yamamoto S, Kozako T, Li HC, Lema C, Yashiki S, Fujiyoshi T, Yoshinaga M, Nagata Y, Akiba S, Takezaki T, Yamada K, Sonoda S. 2004. HTLV-1 provirus load in peripheral blood lymphocytes of HTLV-1 carriers is diminished by green tea drinking. Cancer Sci 95:596–601.

Tachibana N, Okayama A, Ishihara S, Shioiri S, Murai K, Tsuda K, Goya N, Matsuo Y, Essex M, Stuver S, Mueller N. 1992. High HTLV-I proviral DNA level associated with abnormal lymphocytes in peripheral blood from asymptomatic carriers. Int J Cancer 51:593–595.

Tajima K. 1990. The 4th nation-wide study of adult T-cell leukemia/lymphoma (ATL) in Japan: Estimates of risk of ATL and its geographical and clinical features. The T- and B-cell Malignancy Study Group. Int J Cancer 45:237–243.

Taylor GP, Tosswill JH, Matutes E, Daenke S, Hall S, Bain BJ, Davis R, Thomas D, Rossor M, Bangham CR, Weber JN. 1999. Prospective study of HTLV-I infection in an initially asymptomatic cohort. J Acquir Immune Defic Syndr 22:92–100.

van Essen D, Dullforce P, Brocker T, Gray D. 2000. Cellular interactions involved in Th cell memory. J Immunol 165:3640–3646.

Vine AM, Witkover AD, Lloyd AL, Jeffery KJ, Siddiqui A, Marshall SE, Bunce M, Eiraku N, Izumo S, Usuku K, Osame M, Bangham CR. 2002. Polygenic control of human T lymphotropic virus type I (HTLV-I) provirus load and the risk of HTLV-I-associated myelopathy/tropical spastic paraparesis. J Infect Dis 186:932–939.

Wodarz D, Bangham CR. 2000. Evolutionary dynamics of HTLV-I. J Mol Evol 50:448–455.

Wodarz D, Hall SE, Usuku K, Osame M, Ogg GS, McMichael AJ, Nowak MA, Bangham CR. 2001. Cytotoxic T-cell abundance and virus load in human immunodeficiency virus type 1 and human T-cell leukaemia virus type 1. Proc R Soc Lond B Biol Sci 268:1215–1221.

Yamamoto N, Schneider J, Koyanagi Y, Hinuma Y, Hunsmann G. 1983. Adult T-cell leukemia (ATL) virus-specific antibodies in ATL patients and healthy virus carriers. Int J Cancer 15:281–287.

Yao K, Hisada M, Maloney E, Yamano Y, Hanchard B, Wilks R, Rios M, Jacobson S. 2006. Human T lymphotropic virus types I and II western blot seroindeterminate status and its association with exposure to prototype HTLV-I. J Infect Dis 193:427–437.

Yashiki S, Fujiyoshi T, Arima N, Osame M, Yoshinaga M, Nagata Y, Tara M, Nomura K, Utsunomiya A, Hanada S, Tajima K, Sonoda S. 2001. HLA-A*26, HLA-B*4002, HLA-B*4006, and HLA-B*4801 alleles predispose to adult T cell leukemia: The limited recognition of HTLV type 1 tax peptide anchor motifs and epitopes to generate anti-HTLV type 1 tax CD8^+ cytotoxic T lymphocytes. AIDS Res Hum Retroviruses 17:1047–1061.

Yokota T, Cho MJ, Tachibana N, McLane M, Takatsuki K, Lee TH, Mueller M, Essex M. 1989. The prevalence of antibody to p42 of HTLV-I among ATLL patients in comparison to healthy carriers in Japan. Int J Cancer 43:970–974.