In vivo Expression of Human T-lymphotropic Virus Type 1 Basic Leucine-Zipper Protein Generates Specific CD8+ and CD4+ T-Lymphocyte Responses that Correlate with Clinical Outcome

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Background. The roles of the human T-lymphotropic virus type 1 (HTLV-1) basic leucine zipper (HBZ) gene are not clearly understood. We examined CD8+ and CD4+ T cell responses to HBZ and compared these with Tax responses.

Method. Interferon (IFN)-γ and interleukin (IL)-2–secreting T cells were detected by enzyme-linked immunosorbent spot (ELISpot) assays of freshly isolated peripheral blood mononuclear cells (PBMCs) stimulated with synthetic HBZ or Tax peptides. Ten patients with HTLV-1–associated myelopathy (HAM) and 20 asymptomatic HTLV-1 carriers (ACs), (10 high, 10 low viral load).

Results. Of 30 study participants, 17 had detectable HBZ-specific CD4+ T cells and 12 had HBZ-specific CD8+ T cell responses. Detection of Tax-specific CD4+ T cells (IL-2- or IFN-γ-secreting) did not differ by disease status, but Tax-specific CD8+ T cell responses were more commonly detected in patients with HAM. HBZ-specific CD4+ or CD8+ T cells were less likely to be detected than Tax-specific T cells. IL-2-secreting Tax-specific CD8+ T cells, and IFN-γ–secreting Tax-specific CD4+ T cells were associated with HAM. Low viral load, asymptomatic HTLV-1 carriage was associated with IL-2–secreting CD8+ T cells specific for HBZ.

Conclusion. HBZ protein is expressed in vivo in patients with HAM and in ACs. Our results are consistent with the idea that the T cell response to HBZ plays an important part in restricting HTLV-1 viral load.

Human T-lymphotropic virus type 1 (HTLV-1), the first discovered human retrovirus, is prevalent in Japan, the Caribbean, parts of sub-Saharan Africa, South America, Melanesia, and the Middle East, with worldwide prevalence once estimated to be 10–20 million [1]. Infection with HTLV-1 is lifelong. Most subjects remain asymptomatic carriers (ACs), but a significant minority, 7%, will develop adult T cell leukemia/lymphoma (ATLL) [2] or HTLV-1-associated myelopathy (HAM) [3]. The overall risk of disease may reach 10% if HTLV-1–associated uveitis [4], HTLV-1-associated infective dermatitis [5], and other, apparently associated, manifestations such as polymyositis [6], a broad spectrum of lung disease [7], and thyroiditis [8] are included. In addition to these overt diseases, a reduction in life expectancy, of uncertain cause, has been described [5, 9, 10].

HTLV-1, like other retroviruses, contains the structural and enzymatic genes *gag, pro, pol,* and *env* flanked by 2 long terminal repeats (LTR). In addition, HTLV-1 encodes accessory and regulatory proteins in 4 open reading frames (ORFs) I–IV located at the 3′ end of the genome. ORFs III and IV encode 2 well-characterized genes, *tax* and *rex*. Tax appears to play an important role

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in HTLV-1 pathogenesis; it is the first viral protein detected in HTLV-1-expressing cells [11] and has been shown to be the immunodominant antigen recognized by cytotoxic T-lymphocytes (CTL) of patients with HAM and of ACs [12–16].

Recently a new viral protein, HTLV-1 basic leucine zipper (HBZ), has been identified; it is encoded by an ORF located between the *env* and *tax/rex* genes on the minus, or antisense, strand of the HTLV-1 provirus [17]. Transcription of HBZ is controlled by a promoter in the 3′ LTR. HBZ contains a transcription activation domain at the N-terminus and a leucine zipper at the C-terminus and the transcribed RNA has been shown to exist in 3 forms, unspliced HBZ (unHBZ), HBZ spliced-1 (HBZ-SP1), and HBZ spliced-2 (HBZ-SP2) [18, 19]. Unspliced HBZ and HBZ-SP1 have been studied the most, both forms have been detected in multiple HTLV-1 cell lines [18–21] and in peripheral blood mononuclear cells (PBMCs) isolated from patients with ATLL or HAM and from ACs [19–21]. HBZ appears to have biologically important activities at both the RNA and protein levels. HBZ protein inhibits Tax-mediated transactivation of viral transcription from the 5′ LTR through an interaction with JUN and ATF/CREB families [22–25]. HBZ RNA reportedly promotes T cell proliferation [19]. In transgenic mice HBZ promotes CD4+ T cell proliferation, while in inoculated rabbits HBZ enhances infectivity and persistence of HTLV-1 [26]. Similar genes encoded on the antisense strand of HTLV-2, HTLV-3 and HTLV-4 have been described [27–29]. The antisense transcript of HTLV-2 encodes the antisense protein APH-2 that remains able to bind to CREB and to down-regulate Tax-2 dependent expression although APH-2 lacks a leucine zipper domain [28].

We describe the use of an immunological assay to detect evidence of HBZ protein expression in vivo in patients with HAM and in ACs, and report a significant association between the detection of HBZ-specific CD8+ T cell responses with low viral load (LVL) and asymptomatic carriage of HTLV-1. These results are consistent with the conclusion of our recent study of class I binding of HTLV-1 epitopes [30], that the HLA class 1–restricted T cell response plays an important part in limiting HTLV-1 viral load and the risk of HAM.

MATERIALS AND METHODS

Patients and Cells

The patient cohort is based at the National Centre for Human Retrovirology at St Mary's Hospital, London. HTLV-1 infection was confirmed by Western blot and the diagnosis of HAM was made according to World Health Organization criteria [31]. Of 30 patients who participated in this study, 10 had HAM, and 20 were ACs, of whom 10 had HTLV-1 viral loads similar to patients with HAM (ie, >1 HTLV-1 DNA copy/100 PBMCs [%] and referred to as AC high) and 10 had HTLV-1 viral loads <1% (AC low). Uninfected controls were relatives of patients who had been investigated for HTLV-1/2 infection at the Centre and laboratory staff. All patients and controls donated blood for research purposes after giving written informed consent.

Preparation of T Lymphocytes

PBMCs were isolated from fresh whole blood by density gradient centrifugation on Histopaque-1077 (Sigma-Aldrich), washed in phosphate buffer saline (PBS, Sigma-Aldrich), cryopreserved in 10% dimethyl sulphoxide (Sigma-Aldrich) and 90% heat inactivated fetal calf serum (FCS) (Gibco), and stored in liquid nitrogen until use. Cryopreserved cells were thawed and washed with cold PBS/0.5% FCS 3 times. PBMCs were depleted of CD8+ or CD4+ T cells using anti-CD4 or anti-CD8 magnetically labeled beads according to the manufacturer's protocol (Miltenyi Biotec). Depleted PBMCs were washed once with complete medium (CM, RPMI 1640, 10% FCS, 1% penicillin/streptomycin [all from Gibco]). A fraction (10^5) of depleted cells were stained with CD8 and CD4 (Beckman-Coulter) surface markers and analyzed by flow cytometry to determine the percentage of CD4+ and CD8+ T-cells present in each sample.

Peptide Libraries

Peptides spanning Tax (ATK strain, [32]) and HBZ [18, 19, 33] proteins of HTLV-1 were commercially synthesized by Mimotopes Pty Ltd Europe and reconstituted in 50% acetonitrial:molecular-grade water (both Sigma-Aldrich) according to their instructions. HBZ peptides were based on HBZ SP-1 and designed to include the head domain of unspliced HBZ and HBZ SP-2 [18] and the 2 published amino acid polymorphisms, a lysine to glutamic acid at position 41 and isoleucine to threonine at position 99. Peptide purity was determined by reverse phase HPLC and ion spray mass spectroscopy. Tax peptides ($n = 57$) and HBZ peptides ($n = 43$) were overlapping 20-mers offset by 6 amino acids (accession number J02029 for Tax and DQ273132 for HBZ). All peptides used are presented in Table 1.

Enzyme-linked Immunosorbent Spot Assays for Interferon-γ and Interleukin-2

Flat-bottomed 96-well polyvinylidine difluoride (PVDF) membrane-backed plates (MAIPS4510, Millipore) were used. Each well was activated with 15 μL 35% ethanol and washed with sterile PBS. The wells were coated with 100 μL of the primary capture antibody, anti-IFN-γ (mAb clone 1-D1K) or anti-IL-2 (mAb clone IL2-I) (all mAbs from MabTech), each at a concentration of 10 μg/mL. The antibody was allowed to bind overnight at 4°C.

Plates were washed (unless otherwise stated, 6 times with PBS) and blocked with CM for 1 h at room temperature (RT). The blocking solution was discarded and 10^5 cells were added to each well. In CD4+ T cell assays, to duplicate wells stimulatory monoclonal antibodies were added, anti-CD49d (clone HP2/1) and anti-CD28 (clone 28.2) (both from Becton Dickinson), each

Table 1. Peptide Sequences for Tax and HTLV-1 Basic Leucine Zipper Proteins

Tax Peptides	HBZ Peptides Accession No. DQ2732132 (Peptides 1–33)
1 MAHFPGFGQSLLFGYPVYVF	1 MAASGLFRCLPVSCPEDLLV
2 FGQSLLFGYPVYVFGDCVQG	2 FRCLPVSCPEDLLVEELVDG
3 FGYPVYVFGDCVQGDWCPIS	3 SCPEDLLVEELVDGLLSLEE
4 VFGDCVQGDWCPISGGLCSA	4 LVEELVDGLLSLEEELKDKE
5 QGDWCPISGGLCSARLHRHA	5 DGLLSLEEELKDKEEE**K**AVL
6 ISGGLCSARLHRHALLATCP	6 EEELKDKEEE**K**AVLDGLLSL
7 SARLHRHALLATCPEHQITW	7 KEEE**K**AVLDGLLSLEEESRG
8 HALLATCPEHQITWDPIDGR	8 VLDGLLSLEEESRGRLRRGP
9 CPEHQITWDPIDGRVIGSAL	9 SLEEESRGRLRRGPPGEKAP
10 TWDPIDGRVIGSALQFLIPR	10 RGRLRRGPPGEKAPPRGETH
11 GRVIGSALQFLIPRLPSFPT	11 GPPGEKAPPRGETHRDRQRR
12 ALQFLIPRLPSFPTQRTSKT	12 APPRGETHRDRQRRAEEKRK
13 PRLPSFPTQRTSKTLKVLTP	13 THRDRQRRAEEKRKRKKERE
14 PTQRTSKTLKVLTPPITHTT	14 RRAEEKRKRKKEREKEEEKQ
15 KTLKVLTPPITHTTPNIPPS	15 RKRKKEREKEEEKQ**I**AEYLK
16 TPPITHTTPNIPPSFLQAMR	16 REKEEEKQ**I**AEYLKRKEEEK
17 TTPNIPPSFLQAMRKYSPFR	17 KQ**I**AEYLKRKEEEKARRRRR
18 PSFLQAMRKYSPFRNGYMEP	18 LKRKEEEKARRRRRAEKKAA
19 MRKYSPFRNGYMEPTLGQHL	19 EKARRRRRAEKKAADVARRK
20 FRNGYMEPTLGQHLPTLSFP	20 RRAEKKAADVARRKQEEQER
21 EPTLGQHLPTLSFPDPGLRP	21 AADVARRKQEEQERRERKWR
22 HLPTLSFPDPGLRPQNLYTL	22 RKQEEQERRERKWRQGAEKA
23 FPDPGLRPQNLYTLWGGSVV	23 ERRERKWRQGAEKAKQHSAR
24 RPQNLYTLWGGSVVCMYLYQ	24 WRQGAEKAKQHSARKEKMQE
25 TLWGGSVVCMYLYQLSPPIT	25 KAKQHSARKEKMQELGIDGY
26 VVCMYLYQLSPPITWPLLPH	26 ARKEKMQELGIDGYTRQLEG
27 YQLSPPITWPLLPHVIFCHP	27 QELGIDGYTRQLEGEVESLE
28 ITWPLLPHVIFCHPGQLGAF	28 GYTRQLEGEVESLEAERRKL
29 PHVIFCHPGQLGAFLTNVPY	29 EGEVESLEAERRKLLQEKED
30 HPGQLGAFLTNVPYKRIEEL	30 LEAERRKLLQEKEDLMGEVN
31 AFLTNVPYKRIEELLYKISL	31 KLLQEKEDLMGEVNYWQGRL
32 PYKRIEELLYKISLTTGALI	32 EDLMGEVNYWQGRLEAMWLQ
33 ELLYKISLTTGALIILPEDC	33 TSRVRQSVESRLSLGLFRCL[a]
34 SLTTGALIILPEDCLPTTLF	34 SVESRLSLGLFRCLPVSCPE
35 LIILPEDCLPTTLFQPARAP	35 SLGLFRCLPVSCPEDLLVEE
36 DCLPTTLFQPARAPVTLTAW	36 MVNFVSVGLFRCLPVSCPED[b]
37 LFQPARAPVTLTAWQNGLLP	37 VGLFRCLPVSCPEDLLVEEL
38 APVTLTAWQNGLLPFHSTLT	38 DGLLSLEEELKDKEEE**E**AVL[c]
39 AWQNGLLPFHSTLTTPGLIW	39 EEELKDKEEE**E**AVLDGLLSL
40 LPFHSTLTTPGLIWTFTDGT	40 KEEE**E**AVLDGLLSLEEESRG
41 LTTPGLIWTFTDGTPMISGP	41 RKRKKEREKEEEKQ**T**AEYLK[d]
42 IWTFTDGTPMISGPCPKDGQ	42 REKEEEKQ**T**AEYLKRKEEEK
43 GTPMISGPCPKDGQPSLVLQ	43 KQ**T**AEYLKRKEEEKARRRRR
44 GPCPKDGQPSLVLQSSSFIF	
45 GQPSLVLQSSSFIFHKFQTK	
46 LQSSSFIFHKFQTKAYHPSF	
47 IFHKFQTKAYHPSFLLSHGL	
48 TKAYHPSFLLSHGLIQYSSF	
49 SFLLSHGLIQYSSFHSLHLL	
50 GLIQYSSFHSLHLLFEEYTN	
51 SFHSLHLLFEEYTNIPISLL	

Table 1. (*Continued*)

Tax Peptides	HBZ Peptides Accession No. DQ2732132 (Peptides 1–33)
52 LLFEEYTNIPISLLFNEKEA	
53 TNIPISLLFNEKEADDNDHE	
54 LLFNEKEADDNDHEPQISPG	
55 EADDNDHEPQISPGGLEPPS	
56 HEPQISPGGLEPPSEKHFRE	
57 QISPGGLEPPSEKHFRETEV	

NOTE. HTLV-1, human T-lymphotropic virus type 1; HBZ, HTLV-1 basic leucine zipper; VL, viral load; IFN, interferon; IL, interleukin; AC, asymptomatic HTLV-1 carriers.

[a] Peptides 33–35 HBZ-SP2 head domain (Cavanagh et al [18]).

[b] Peptides 36–37 unspliced HBZ head domain (Cavanagh et al [18]).

[c] Peptides 38–40 amino acid change (in bold) from peptides 5–7 (accession no. AB219938).

[d] Peptides 41–43 amino acid change (in bold) from peptides 15–17 (accession no. AB219938).

at a final concentration of 0.5 μg/mL, and peptide pools (either the Tax pool or the HBZ pool) containing each peptide at a final concentration of 2 μmol/L, in a total volume of 100 μL CM. CD8+ T cell assays were prepared in the same way except for the omission of anti-CD49d mAb [13]. To induce nonspecific cytokine production by PBMCs depleted of CD8+ T cells, we added 0.1 ng/mL phorbol myristate acetate (PMA) and 0.5μg/mL calcium ionophore A23187 (both from Sigma-Aldrich) to the positive control wells. The plates were incubated at 37°C in 5% CO_2 for 6 h.

After incubation, the cells were discarded and the plate washed. A second-layer biotinylated antibody specific to either IFN-γ (clone 7-B6-1) or IL-2 (clone IL2-II) at 1μg/mL in .5% FCS in 100 μL PBS was added for 2 h at RT. Excess antibody was discarded and the plate washed. Streptavidin alkaline phosphatase conjugate (1:1000 dilution in 100 μL PBS/0.5% FCS) was added for 1 h at RT. The solution was discarded and the plate washed. Chromogenic alkaline phosphatase substrate (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate [NBT/BCIP], Europa Bioproducts) was added and placed the plates for 10–15 min at RT in the dark after which the reaction was terminated by washing the plates with tap water. Plates were allowed to dry and the number of spots per well were counted using AID 3.5 software (AID ELIspot reader, Germany). A response was designated positive if the number of spots exceeded the mean + 2 standard deviations (SDs) of the spot count in the negative wells (no peptide added). The frequencies of cytokine-producing CD8+ T cells or CD4+ T cells are presented as the number of spots per 10^5 CD8+ or CD4+ T cells [34].

Human T-Lymphotropic Virus Type 1 Viral Load Quantification

Genomic DNA was extracted from PBMCs using QIAamp DNA mini kit (Qiagen). DNA extracted from MT-2 cell lines was serially diluted 10-fold to generate standard curves ranging from 2 to 20,000 β-globin copies and 7 to 70,000 HTLV-1 tax copies. Real time polymerase chain reaction (PCR) was performed using LightCycler FastStart DNA Master^{PLUS} SYBR Green 1 (Roche), 0.6U LightCycler Uracil DNA Glycosylase (Roche, Germany), 5 pmol of each forward (F) and reverse (R) primer for Tax (F: 5′-CGGATACCCAGTCTACGTGT-3′, R: 5′-GAG CCGATAACGCGTCCATCG-3′) and β-globin (F: 5′-GCAA GGTGAACGTGGATG-3′, R: 5′-TAAGGGTGGAAAATTGA CC-3′), 750 ng - 7.5 ng DNA per reaction, in a Roche LightCycler 1.5. Thermal cycler conditions were: denaturation at 40°C for 8 min and 95°C for 10 min, followed by 45 amplification cycles of 95°C for 10 s, 58°C for 5 s, 72°C for 8 s, and a single 10 s acquisition at 85°C for Tax and 81°C for β-globin. A final melting curve was performed with continuous acquisition between 60 and 95°C. HTLV-1 DNA copy number was calculated from the standard curves and standardized to the number of β-globin copies divided by 2 and reported as HTLV-1 DNA copies/100 PBMCs (%).

Statistical Analysis

Data were analysed in GraphPad PRISM, version 5.01 (GraphPad software) using nonparametric statistical tests to limit the assumptions made about the distribution of the data. Categorical comparisons of Tax and HBZ responses were made using the χ^2 test, and Fisher exact test was used for comparison between clinical states. The Mann–Whitney *U* test was used to compare continuous variables.

RESULTS

Human T-Lymphotropic Virus Type 1 Viral Load (Table 2)

As per the study design, there was no statistical significance in the median viral load between patients with HAM (7.17%, range 3.12%–46.14%) and AC high (8.23%, range 1.92%–11.72%), whereas AC low (.115%, range .001%–.72%) had significantly lower viral loads than AC high or patients with HAM ($P = .002$ in each case; Mann–Whitney *U* test).

Table 2. Human T-Lymphotropic Virus Type 1 (HTLV-1) Tax and HTLV-1 Basic Leucine Zipper–Specific $CD8^+$ and $CD4^+$ T Cell Responses in HTLV-1–Infected Subjects

		CD8+ T cell IFN-γ responses[b]		CD8+ T cell IL-2 responses[b]		CD4+ T cell IFN-γ responses[b]		CD4+ T cell IL-2 responses[b]	
Subjects	VL[a]	Tax	HBZ	Tax	HBZ	Tax	HBZ	Tax	HBZ
HAM									
TCG	7.69	45	14	0	0	232	0	210	0
TAL	46.15	46	0	44	0	19	4	16	9
TCL	6.7	295	0	39	0	5	0	0	0
TCO	4.91	0	0	2	0	31	0	17	0
TBU	3.12	111	0	2	0	21	12	12	0
TCQ	13.79	0	0	2	9	23	5	17	0
TBO	13.87	763	0	30	0	5	0	0	0
TAA	6.87	814	0	7	0	0	0	0	0
TCF	7.17	24	0	4	0	0	0	0	0
TBW	15.83	7	3	10	3	15	0	46	14
Median	**7.17**	**79**	-[c]	**7**	-[c]	**20**	**5**	**17**	-[c]
AC high									
HCH	9.5	0	0	0	0	63	0	0	0
HEZ	5.1	80	0	0	0	5	4	5	4
HES	11.4	44	81	0	0	18	0	7	7
HEI	3.4	0	20	0	0	4	0	17	6
HBX	11.25	0	0	0	0	64	27	15	5
HDR	1.92	77	17	0	0	0	3	9	0
HCP	11.72	0	0	0	0	0	0	0	0
HAY	8.58	0	0	0	0	0	0	6	0
HBT	2.44	292	0	13	0	17	0	2	0
HBE	7.88	5	0	1	0	0	0	0	0
Median	**8.23**	**77**	**20**	-[c]	**ND**[d]	**5**	**4**	**6**	**6**
AC low									
HBA	0.001	0	19	0	6	0	0	0	0
HDH	0.13	97	0	0	0	0	0	0	0
HDN	0.72	177	0	5	5	18	7	12	33
HBV	0.01	0	0	78	0	8	0	43	8
HAE	0.03	17	23	0	0	26	2	0	0
HCL	0.17	246	3	53	5	0	0	4	0
HFL	0.1	2	2	0	0	0	0	0	2
HX	0.09	0	0	0	0	0	0	0	1
HAD	0.47	277	70	87	7	22	21	5	0
HDT	0.23	0	0	0	0	7	0	7	8
Median	**0.115**	**137**	**19**	**66**	**6**	**18**	**7**	**7**	**8**

NOTE. IFN, interferon; IL, interleukin; HAM, HTLV-1-associated myelopathy; AC, asymptomatic HTLV-1 carriers; HBZ, HTLV-1 basic leucine zipper.

[a] Data presented as spot-forming cells per 1E5 CD8 or CD4 T cells for each cytokine measured.

[b] Viral load is presented as HTLV-1 Tax DNA copies per 100 PBMCs.

[c] No median value calculated.

[d] Not detected.

Detection of Any Tax or HBZ Immune Responses (Table 2, 3A and 3B)

Tax-specific T cell responses were detected in all patients with HAM. Although 2 out of 10 patients lacked a detectable CD4+ T cell response to Tax, usually both IFN-γ and IL-2 responses were detected in the same patient. Tax-specific CD8+ T cells were also detected in most HTLV-1 asymptomatic carriers, but CD8+ T cell IL-2 responses, and hence dual responses, were significantly less common in symptomatic carriers. However, this was driven by ACs with high viral load in whom Tax-specific IL-2 CD8+ T cell responses were least common. Furthermore, CD8+ T cell Tax responses differed by clinical condition

Table 3. Number of Subjects with Detectable Interferon-γ or Interleukin-2 Response to Tax and HBZ

A) Tax

	CD8+				CD4+			
	IFN- γ	IL-2	Dual response[a]	Any response[b]	IFN- γ	IL-2	Dual response[a]	Any response[b]
HAM ($n = 10$)	8	9	7	10	8	6	6	8
AC high ($n = 10$)	5	2 ($P = .003$)[c]	2 ($P = .03$)[c]	5 ($P = .02$)[c]	6	7	6	8
AC low ($n = 10$)	6	4 ($P = .03$)[c]	3	7	5	5	3	6
HAM ($n = 10$)	8	9	7	10	8	6	6	8
AC ($n = 20$)	11	6 ($P = .003$)[c]	5 ($P = .02$) [c]	12 ($P = .02$) [c]	11	12	9	14
HVL[d] patients ($n = 20$)	13	11	9	15	14	13	12	16
LVL[e] patients ($n = 10$)	6	4	3	7	5	5	3	6

B) HBZ

	CD8+				CD4+			
	IFN-γ	IL-2	Dual response[a]	Any response[b]	IFN-γ	IL-2	Dual response[a]	Any response[b]
HAM ($n = 10$)	2	2	1	3	3	2	1	4
AC high ($n = 10$)	3	0	0	3	3	4	2	5
AC low ($n = 10$)	5	4 ($P = .04$)[c]	3	6	3	5	1	7
HAM ($n = 10$)	2	2	1	3	3	2	1	4
AC ($n = 20$)	8	4	3	9	6	9	3	12
HVL[d] patients ($n = 20$)	5	2	1	6	6	6	3	11
LVL[e] patients ($n = 10$)	5	4 ($P = .04$)[c]	3	6	3	5	1	7

NOTE. HBZ, HTLV-1 basic leucine zipper; IFN, interferon; IL, interleukin; HAM, HTLV-1-associated myelopathy; AC, asymptomatic HTLV-1 carriers; HVL, high viral load; LVL, low viral load.

[a] Dual response refers to patients with BOTH IFN-γ and IL-2 responses and not to cells that express both cytokines.

[b] Any response refers to patients with EITHER IFN-γ or IL-2 response.

[c] Fisher exact test 1 tail *P* values quotes when significant and they refer to the values above the parenthesis.

[d] HVL: High viral load patients are patients with HAM and AC with viral load >1%.

[e] LVL: Low viral load patients refers to asymptomatic carriers with viral load <1%.

and not by viral load. Tax-specific CD4+ T cell responses significantly differed between AC with low viral load and patients with HAM or ACs with high viral load regardless of whether IFN-γ or IL-2 responses were considered (Table 3A).

HBZ-specific CD4+ T cell responses were detected in a minority of patients with no significant difference between ACs high, ACs low or patients with HAM regardless of cytokine (IFN-γ or IL-2). HBZ responses were much less likely to be detected than Tax-specific CD8+ T cell responses. However, HBZ-specific CD8+ T cell IL-2 responses were significantly more commonly detected in AC with low viral load than patients with high viral load ($P = .04$).

We next examined the size of the response, where detected, by quantifying the number of spot-forming cells specific for HBZ or Tax (Table 4). CD8+ T cell Tax responses detected in patients with HAM and in ACs with high viral load were predominantly IFN-γ secreting. However, when detected the absolute number of Tax-specific CD8+ IL-2–secreting SFC was higher in AC low than in subjects (patients with HAM and AC high) with high viral load, although they remained fewer than IFN-γ–producing Tax-specific CD8+ T cells.

CD4+ T cell Tax responses were numerically less than CD8+ T cell Tax responses with no significant difference between patient categories.

The size of the HBZ responses was generally smaller than the Tax responses (comparing the number of spot forming cells [SFC]) for both CD4+ T cells and CD8+ T cells and did not differ by cytokine secreted or disease state.

DISCUSSION

HBZ is Expressed In Vivo; T Cell Response to HBZ is Associated with Low Viral Load and Asymptomatic Carriage

Detection of HBZ-specific CD4+ and CD8+ T cell responses ex vivo in these short-term ELISpot assays implies expression of this protein in vivo in asymptomatic carriers as well as in patients with HAM. Importantly, CD8+ T cell responses to HBZ were found significantly more frequently in patients with low HTLV-1 viral load. This accords with our recent immunogenetic study [30], in which HLA Class 1 genotypes that predicted efficient T cell recognition of HBZ were associated with low viral load. In our study, 4 of 10 ACs with a low viral load (median,

Table 4. Median Frequency and Range of Tax- or HBZ-Specific CD8+ and CD4+ T Cells

	Tax				HBZ			
	CD8		CD4		CD8		CD4	
	IFN-γ	IL-2	IFN-γ	IL-2	IFN-γ	IL-2	IFN-γ	IL-2
HAM (n = 10)	79 (7–814)	7 (2 - 44)	20 (5–232)	17 (12–210)	-[a] (3 and 14)	-[a] (3 and 9)	5 (4–12)	-[a] (9 and 14)
AC high (n = 10)	77 (5–295)	-[a] (1 and 13)	5 (4–46)	6 (2–17)	20 (17–81)	ND[b]	4 (3–27)	6 (4–7)
AC low (n = 10)	137 (2–277)	66 (5–87)	18 (7–26)	7 (4–43)	19 (2–70)	6 (5–7)	7 (2–21)	8 (1–33)
All participants (n = 30)	80 (2–814)	10 (1–87)	18 (4–232)	12 (2- 210)	18 (2–81)	6 (3–9)	7 (2–27)	7 (1–33)
HAM (n = 10)	79 (7–814)	7 (2 - 44)	20 (5–232)	17 (12–210)	-[a] (3 and 14)	-[a] (3 and 9)	5 (4–12)	-[a] (9 and 14)
AC (n = 20)	80 (2 -295)	33 (1–87)	28 (4–46)	7 (2–43)	20 (2–81)	6 (5–7)	6 (2–27)	6 (1–33)
HVL[c] patients (n = 20)	77 (5–814)	7 (1–44)	19 (4–232)	15 (2–210)	19 (3–81)	6 (3–9)	5 (3–27)	7 (4–14)
LVL[d] patients (n = 10)	137 (2–277)	66 (5–87)	18 (7–26)	7 (4–43)	19 (2–70)	6 (5–7)	7 (2–21)	8 (1–33)

NOTE. HBZ, HTLV-1 basic leucine zipper; IFN, interferon; IL, interleukin; HAM, HTLV-1-associated myelopathy; AC, asymptomatic HTLV-1 carriers; HVL, high viral load; LVL, low viral load.

Median frequency and in parenthesis the range of frequencies

[a] No median value calculated.

[b] Not detected.

[c] HVL: High viral load patients with viral load more than 1%, which includes patients with HAM and AC.

[d] LVL: Low viral load patients with viral load less than 1% and only includes asymptomatic carriers.

.1%) had a detectable CD8+ T cell IL-2 response to HBZ compared with none of the 10 ACs with a high viral load (median, 8.2%; Fisher exact test $P = .08$). Suemori et al reported that although HBZ can be presented by HLA-A*0201, HBZ-specific CTLs were not able to discriminate between HTLV-1-infected and uninfected cells because HBZ is expressed at low levels. In their study, HBZ-specific CTLs were scarcely detectable using HLA-A*0201/HBZ $_{26\text{-}34}$ tetramer analysis on PBMCs from 6 HLA-A*0201-positive patients, 5 with ATLL and 1 AC [35].

Previously, the Tax protein has been shown to be immunodominant in the CD8+ T cell response to HTLV-1 [13]. Our finding that Tax-specific responses were detected in all patients with HAM and in most asymptomatic carriers is consistent with previous reports [13, 34]. Furthermore, Tax-specific responses (both IFN-γ and IL-2) were found in both CD4+ and CD8+ T cell populations, and, when tax responses were present, the frequency of responding cells was high compared with HBZ-specific T cells. There is considerable interest in whether it is the frequency (ie, the numerical size of the response) or the efficacy of the response (as measured by lysis of infected cells) that is important in controlling viral replication [36]. Like Suemori et al [35], we find, where detected at all, a very low frequency of HBZ specific CD8+ T cells. However, our comparison of patient categories both by disease and by viral load leads us to conclude that even a very low frequency response to a critical target antigen may contribute significantly to the control of viral replication.

IL-2 Responses May Be More Protective than IFN-γ Responses

A previous study has suggested that IL-2 does not contribute to initial antigen-stimulated T cell activation but is necessary for the survival of the activated T cells and the generation of memory T cells [37]. We found that the presence of any detectable CD8+ T cell response to Tax (ie, IL-2 or IFN-γ) was associated with HAM, independently of viral load, whereas an IL-2 response to HBZ was associated with low viral load. In some ACs, IL-2 responses in the absence of an IFN-γ response could be detected, whereas in patients with HAM IL-2 responses were detected only when an IFN-γ response was present. This suggests that following HBZ recognition re-exposure of a lymphocyte or its progeny leads to activation of the IL-2 secreting T cell and better infection control, ie, a protective response. We hypothesise that a low level of HTLV-1 HBZ antigen expression is associated with an efficient immune response, re-exposure of HBZ-specific CD8+ T cells or their progeny is treated as a secondary infection with IL-2 production and this results in asymptomatic carriage. This would be similar to the situation in *Mycobacterium tuberculosis* infection wherein IL-2–secreting cells were more common in latent infection than in active *M. tuberculosis* [38]. Conversely, we speculate that in the absence of this response HTLV-1 antigen exposure is repeatedly seen as a new infection leading to high levels of the IFN-γ and thus is found in the patients with inflammatory disease. Therefore, HBZ-specific CD8+ T cells secreting IL-2 would be associated with asymptomatic carriage, whereas Tax-specific CD8+ T cell secreting IFN-γ would be associated with an increased risk of disease.

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