MAJOR ARTICLE

# Human T Cell Lymphotropic Virus (HTLV) Type–1–Specific CD8$^+$ T Cells: Frequency and Immunodominance Hierarchy

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**Human T cell lymphotropic virus type 1 (HTLV-1) causes HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP). We used interferon-γ enzyme-linked immunospot assays with overlapping peptides spanning the entire HTLV-1 proteome to test whether the HTLV-1–specific CD8$^+$ T cells differed significantly in frequency or immunodominance hierarchy between patients with HAM/TSP and asymptomatic carriers and whether the frequency correlated with provirus load. Tax was the immunodominant target antigen. There was no significant qualitative or quantitative difference in the HTLV-1–specific CD8$^+$ T cell response between the 2 groups. Virus-specific CD8$^+$ T cell frequency alone does not indicate the effectiveness of the cytotoxic T lymphocyte response in controlling provirus load at equilibrium.**

Human T cell lymphotropic virus type 1 (HTLV-1) is the etiological agent of both adult T cell leukemia and chronic inflammatory disease, of which HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP) is the most commonly recognized. The CD8$^+$ T cell response to HTLV-1 has been proposed to contribute to the pathogenesis of HAM/TSP for the following reasons. In some studies, a higher chronically activated CD8$^+$ T cell response that is specific to HTLV-1 has been found in patients with HAM/TSP, compared with asymptomatic carriers [1–3], but other investigators have found that the differences were not significant [4–6]. The association of high levels of cytotoxic T lymphocytes (CTLs) with HAM/TSP disease is potentially important, because it suggests that CTLs could be directly involved in the causation of disease. HTLV-1–specific CD8$^+$ T cells, in addition to CD4$^+$ T cells, have been found in CSF and in HAM/TSP lesions [7]. However, the evidence that HTLV-1–specific CD8$^+$ T cells contribute to the pathogenesis of HAM/TSP remains circumstantial.

There has been no systematic analysis of the immunodominance hierarchy of the CD8$^+$ CTL response to the full range of antigens of HTLV-1. Thus, the aims of the present study were to test the hypotheses that (1) the total frequency and/or the immunodominance hierarchy of interferon (IFN)–γ–producing HTLV-1–specific CD8$^+$ T cells were significantly different between patients with HAM/TSP and asymptomatic carriers and (2) the frequency of HTLV-1–specific CD8$^+$ T cells was positively correlated with provirus load in either patients with HAM/TSP or asymptomatic carriers.

## MATERIALS AND METHODS

Subjects were informed and consenting HTLV-1-infected asymptomatic carriers and patients with HAM/TSP who were attending the HTLV-1 clinic at the

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National Referral Centre for Human Retrovirology at St. Mary's Hospital, Imperial College, London. Uninfected control subjects were healthy, uninfected laboratory staff. HTLV-1 infection was confirmed by the presence of antibodies to HTLV-1 Gag (p19 and p24) and Env (rgp21 and rgp46-I) antigens in serum samples, by use of Western blot (HTLV 2.4; Genelabs). The diagnosis of HAM/TSP was made according to World Health Organization criteria. Peripheral blood mononuclear cells (PBMCs) were isolated and cryopreserved as described elsewhere [8]. Ethical permission was obtained from the local research ethics committee of St. Mary's Hospital, London.

After being thawed and washed twice in cold sterile PBS, PBMCs were depleted of $CD4^+$ T cells by use of magnetic microbeads (Miltenyi Biotec), according to the manufacturer's instructions, and run through 2 successive columns, to achieve maximum depletion. Typically, there were <1% $CD4^+$ T cells left after depletion (data not shown). For certain control experiments, further serial depletions of $CD56^+$, $CD16^+$, or $CD8^+$ cells were also performed by use of magnetic microbeads (Miltenyi Biotec), according to the manufacturer's instructions, before use in the ELISPOT assay.

The quantification of HTLV-1 proviral DNA and the synthetic peptide libraries used have been described elsewhere [9]. Peptides were grouped in pools of 15–30 (mean, 22; the peptides from the Gag sequence were kept in 3 pools that corresponded to Gag p15, p19, and p24) and added to the cell-culture medium to achieve a final concentration of 10 $\mu$g/mL (~5 $\mu$mol/L) each peptide before incubation at 37°C. Methods and analyses for the IFN-$\gamma$ ELISPOT assays and associated flow cytometry were as described elsewhere [8] and were adapted for $CD8^+$ work by depleting PBMCs of $CD4^+$ cells. In all experiments, 20 nmol/L concanamycin A (an inhibitor of perforin release) was added to the culture medium, to prevent CTL-mediated lysis via the perforin-dependent cytotoxic pathway. An anti-CD28 costimulatory monoclonal antibody (at 0.5 $\mu$g/mL, clone CD28.2; Pharmingen) was added. All assays were performed in duplicate wells.

## RESULTS

NK cells and T cells (both $CD4^+$ [Th1 type] and $CD8^+$) are the respective primary producers of IFN-$\gamma$ during the innate and adaptive phases of immune response to virus infection. To confirm that the responding cells were $CD8^+$ T cells, we therefore undertook experiments with serial depletion of $CD4^+$ followed by $CD56^+$ and $CD16^+$ cells (to remove the NK cell population). $CD8^+$ cells were depleted as controls. Figure 1*A* shows photographs of representative $CD8^+$ ELISPOT wells. Figure 1*B* shows that the response was eliminated when both CD4 and CD8 depletions were performed, but that CD4 depletion, followed by CD16 and CD56 depletion, did not affect the response. These experiments confirmed that the responding subset in these assays was $CD8^+$ T cells.

We then used this short-term ELISPOT assay to study samples from 10 patients with HAM/TSP, 7 asymptomatic carriers, and 3 uninfected control subjects with the entire library of peptides that represent the HTLV-1 proteome. The 3 subject groups did not differ in median age ($P > .1$, 2-tailed Mann-Whitney *U* test): patients with HAM/TSP had a median age of 70.5 years; asymptomatic carriers, 60.0 years; and uninfected control subjects, 48.0 years (table 1).

The data in table 1 show that Tax was the most commonly recognized antigen in the $CD8^+$ T cell response (16/17 infected subjects), and an anti-Tax response was found in all but 1 subject (HAE), usually at high frequency. However, the data show that there were variable responses to other HTLV-1 proteins, and only 1 subject (TAQ) recognized only Tax. The next most frequently recognized proteins were Pol (12/17), Env (11/17), Gag (10/17), Rof (6/17), Tof (5/17), Pro (3/17), and Rex (1/17). Tax also elicited $CD8^+$ T cell frequencies (median, 4700 sfcs/$10^6$ $CD8^+$ T cells [14mers]) that were significantly larger than the proteins Pol (median, 900 sfcs/$10^6$; $P = .0152$), Env (median, 600; $P = .0015$), and Gag (median, 200 sfcs/$10^6$; $P < .0001$, 2-tailed Mann-Whitney *U* test). As expected, samples from uninfected control subjects did not show detectable positive responses (>2 SD of the mean negative) to HTLV-1 peptides.

We compared the frequency of IFN-$\gamma$–secreting Tax-specific $CD8^+$ T cells, using 13mer and 20mer peptides covering all of Tax, to test whether 20mer peptides detected a lower frequency of HTLV-1–specific $CD8^+$ T cells. The data in table 1 show that the estimates of specific $CD8^+$ T cell frequency elicited by Tax peptides were not significantly different (median for paired 13mers, 6600 sfcs/$10^6$; median for paired 20mers, 3400 sfcs/$10^6$; $P = .0830$, 2-tailed Wilcoxon matched-pairs signed rank sum test), although, with 20mer peptides, the tendency was toward a lower frequency. The estimate of total HTLV-1–specific $CD8^+$ T cell frequency was not significantly different between patients with HAM/TSP and asymptomatic carriers (patients with HAM/TSP, median, 6600 sfcs/$10^6$; asymptomatic carriers, median, 9500 sfcs/$10^6$; $P = .4747$, 2-tailed Mann-Whitney *U* test). Tax-specific $CD8^+$ T cell frequencies in patients with HAM/TSP (median, 4700 sfcs/$10^6$) were, in fact, lower than those in asymptomatic carriers (median, 6400 sfcs/$10^6$) when Tax 13mer data were used (table 1), although this difference was not statistically different ($P = .4278$, 2-tailed Mann-Whitney *U* test).

The provirus loads in these patients with HAM/TSP (median, 12.45%) were significantly higher than those in asymptomatic carriers (median, 1.20%) ($P = .0330$, 2-tailed Mann-Whitney *U* test). This represented an increase of >10-fold and showed that a high $CD8^+$ T cell frequency was not associated with

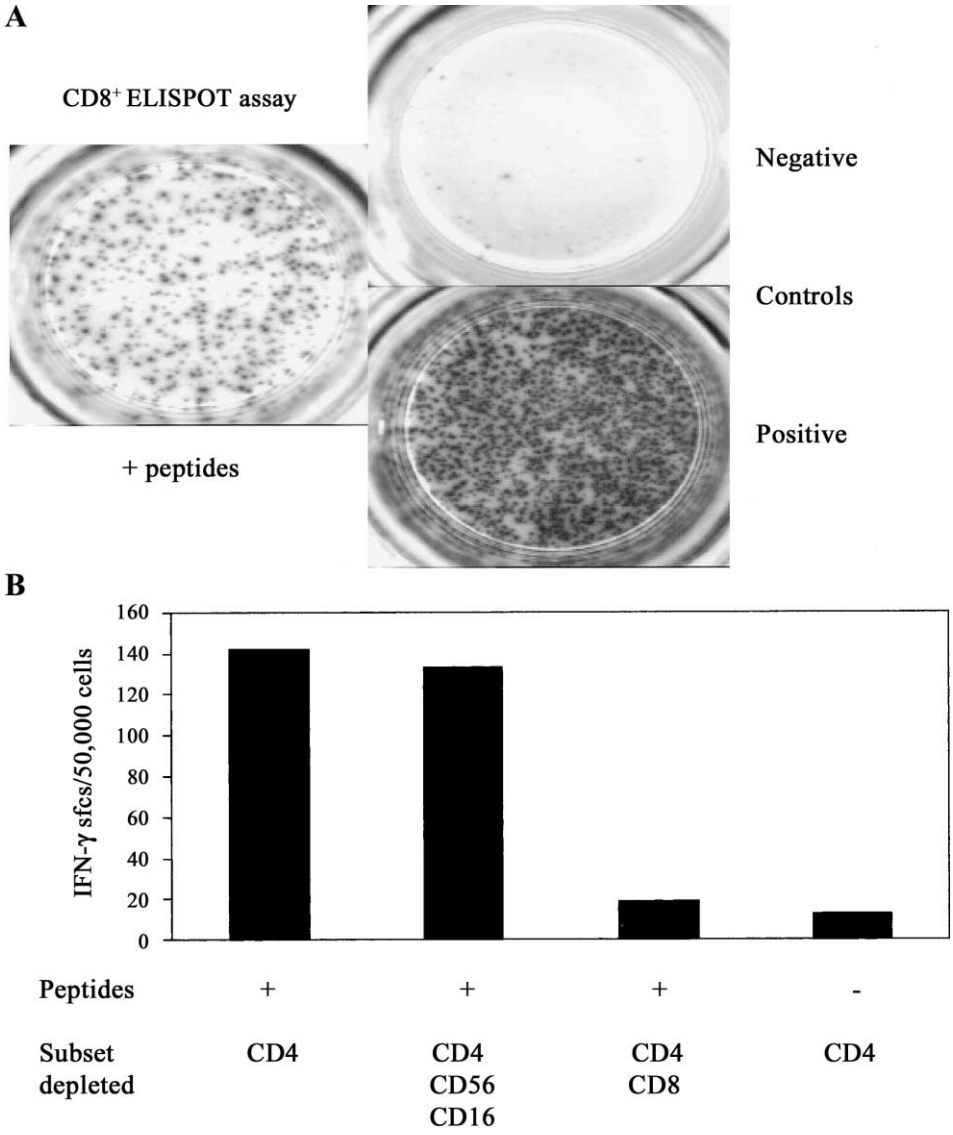


**Figure 1.** *A,* Examples of digital photographs of $CD8^+$ ELISPOT wells. *B,* Interferon (IFN)–γ responses from the $CD8^+$ T cell subset and not the NK cell subset. Serial depletions of CD4, followed by either CD16 and CD56 or CD8 depletion, showed that responses were eliminated by depletion of $CD4^+$ and $CD8^+$ cells but not by the depletion of $CD16^+$ and $CD56^+$ cells, and these responses were induced by peptides. The mean no. of spot-forming cells (sfcs) of 2 experiments is shown.

HAM/TSP disease status. This observation contrasted strongly with the results of our recent data on HTLV-1–specific $CD4^+$ T cell frequencies and disease status [8, 9]. The immunodominant antigen for HTLV-1–specific $CD4^+$ T cells has been found to be Env [10].

We analyzed the ELISPOT data for any correlation between the frequency of either Tax-specific or total HTLV-1–specific $CD8^+$ T cells and the provirus load from the same blood sample. The data showed weak correlations (not statistically significant) between frequencies of IFN-γ–secreting HTLV-1–specific $CD8^+$ T cells and provirus load in patients with HAM/TSP (Tax-specific and total HTLV-1–specific $CD8^+$ T cell frequency vs. provirus load; 2-tailed $P = .5135$ for both, Spearman rank correlation test) and asymptomatic carriers (Tax-specific and total HTLV-1–specific $CD8^+$ T cell frequency vs. provirus load; $P = .6615$ and $P = .9635$, respectively, Spearman rank correlation test).

## DISCUSSION

We have reported here a systematic analysis of the entire HTLV-1–specific IFN-γ–secreting $CD8^+$ T cell population that is present in HTLV-1-infected subjects, regardless of HLA genotype. We confirmed that $CD8^+$ T cells are the primary subset of IFN-γ secreting cells that respond to HTLV-1 peptides (in $CD4^+$-depleted PBMCs) during a short-term (6-h) assay. We then

**Table 1. Immunodominance hierarchy of the human T cell lymphotropic virus type (HTLV)–1–specific CD8⁺ T cell response.**

| Group, subject | Age, years | Provirus load, % | Peptide | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Tax13 | Tax20 | Env | Rex | Tof | Rof | Gag | Pol | Pro | Total |
| HAM/SP | | | | | | | | | | | | |
| TAY | 78 | 14.00 | 8480 | ND | 5590 | 0 | 1540 | 0 | 3000 | 7880 | 0 | 26,500 |
| TBA | 63 | 10.90 | 631 | 496 | 2000 | 0 | 996 | 673 | 0 | 902 | 0 | 5220 |
| TAQ | 75 | 1.50 | 584 | ND | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 584 |
| TAU | 73 | 2.70 | 534 | ND | 300 | 152 | 243 | 265 | 117 | 2350 | 0 | 3961 |
| TBI | 68 | 32.00 | 3390 | ND | 0 | 0 | 0 | 0 | 0 | 1230 | 0 | 4620 |
| TW | 38 | 23.00 | 6620 | 3450 | 980 | 0 | 0 | 0 | 392 | 0 | 0 | 7990 |
| TBK | 78 | 35.20 | 4900 | 2390 | 2060 | 0 | 0 | 0 | 416 | 537 | 416 | 8330 |
| TAN | 47 | 6.20 | 29,000 | 34,000 | 3300 | 0 | 0 | 0 | 0 | 0 | 0 | 37200 |
| TAE | 80 | 3.10 | 7850 | 8220 | 337 | 0 | 214 | 241 | 228 | 296 | 0 | 9170 |
| TAL | 66 | 26.20 | 4460 | 3160 | 617 | 0 | 0 | 0 | 225 | 0 | 0 | 5300 |
| AC | | | | | | | | | | | | |
| HBD | 49 | 0.01 | 8130 | 6430 | 0 | 0 | 0 | 0 | 394 | 947 | 0 | 9470 |
| HBE | 70 | 11.50 | 8960 | 6160 | 2170 | 0 | 0 | 563 | 2290 | 883 | 374 | 15000 |
| HBF | 37 | 6.50 | 857 | 690 | 0 | 0 | 0 | 290 | 273 | 623 | 0 | 2040 |
| HT | 75 | 1.20 | 15,900 | 5300 | 1680 | 0 | 0 | 0 | 0 | 4940 | 0 | 22520 |
| HAY | 61 | 10.60 | 4110 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1570 | 5680 |
| HX | 60 | 0.01 | 4670 | 2960 | 772 | 0 | 2320 | 822 | 1810 | 4530 | 0 | 14924 |
| HAE | 57 | 0.01 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5690 | 0 | 5690 |
| Un | | | | | | | | | | | | |
| Un1 | 40 | NA | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Un2 | 48 | NA | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Un3 | 65 | NA | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

**NOTE.** Patients with HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP) have anonymized codes beginning with T, asymptomatic HTLV-1 carriers (ACs) have codes beginning with H, and uninfected control subjects have codes beginning with Un. All results shown are the mean of replicate well counts minus the mean negative (background) and are expressed in no. of spot-forming cells/$10^6$ CD8⁺ T cells. Counts were considered to be positive if values were ⩾(2 SD + mean negative [mean of a minimum of 6 negative wells]). All non-Tax peptides are 20mers. 0, below the limit of detection; NA, not applicable; ND, not done; Tax13, Tax 13mer peptides (overlapping by 9); Tax20, Tax 20mer peptides (overlapping by 14).

confirmed that Tax was indeed the immunodominant antigen in the CD8⁺ T cell response in HTLV-1–infected subjects, both patients with HAM/TSP and asymptomatic carriers [11]. Tax elicited the highest frequencies of CD8⁺ T cells in both groups of HTLV-1–infected subjects (except in 1 asymptomatic carrier), and it was also the most frequently recognized HTLV-1 antigen in both groups. Pol, Env, and Gag were the next most frequently recognized antigens, but, between the 2 groups, there were no significant differences found in terms of the frequency of response of CD8⁺ T cells to any single antigen.

Previous researchers have reported that HTLV-1 antigens other than Tax elicit responses in the CD8⁺ T cell compartment [12, 13]. One of these studies [13] showed that asymptomatic carriers had detectable responses to Tof and Rof, but they were unable to detect responses in patients with HAM/TSP, perhaps because of high background responses. We detected responses to Rof and Tof epitopes in certain patients with HAM/TSP, as well as in some asymptomatic carriers. These observations suggest that several HTLV-1 proteins are persistently expressed in vivo and that they elicit the chronically activated CTL response. We have also shown that there is no significant difference in the CD8⁺ T cell antigen specificity or immunodominance hierarchy between patients with HAM/TSP and asymptomatic carriers. Furthermore, a direct comparison (in the same assay) between the 13mers and 20mers of Tax peptides showed that either length of peptide could be used to estimate the frequencies of Tax-specific CD8⁺ T cells. Therefore, we were able to extrapolate these results and use the data for other non-Tax viral proteins derived from 20mers, to estimate the individual HTLV-1 protein–specific and total frequencies of HTLV-1–specific CD8⁺ T cells.

Other groups have found that the frequency of anti-HTLV–1 CD8⁺ T cells is significantly higher in patients with HAM/TSP than in asymptomatic carriers and that this frequency is correlated positively with provirus load and with the severity of disease [1, 3, 14]. It has therefore been suggested that HTLV-

1–specific CD8$^+$ T cells are the main subset of pathogenic lymphocytes in HAM/TSP [15]. Work from this laboratory has shown that the frequency of such cells in PBMCs is not significantly different between the groups, according to the results of chromium release assays [5], limiting dilution analysis [4], and major histocompatibility complex class I tetramer analysis [6, 16]. This conclusion is now further supported by the results of our IFN-$\gamma$ ELISPOT assay and was true for both Tax-specific CD8$^+$ T cells alone and total HTLV-1–specific CD8$^+$ T cells.

We have shown that the total estimated anti–HTLV-1 CD8$^+$ T cell frequency did not differ significantly between patients with HAM/TSP and asymptomatic carriers, even though the provirus loads were 10-fold higher in the HAM/TSP group. Furthermore, there were no significant correlations between provirus load and anti–HTLV-1 CD8$^+$ T cell frequency in either group. Conflicting data on the correlation between specific CD8$^+$ T cell frequencies and virus load have also been reported in work on HIV-1.

Using 3 different assays (limiting dilution analysis with chromium release assay, HLA class I tetramer binding analysis, and IFN-$\gamma$ ELISPOT assay), we have studied the frequency of HTLV-1–specific CD8$^+$ T cells in 17 patients with HAM/TSP and 20 asymptomatic carriers in the United Kingdom and in 19 patients with HAM/TSP and 19 asymptomatic carriers in Japan. The findings are consistent with the hypotheses that (1) the specific CD8$^+$ T cell frequency differs little, if at all, between patients with HAM/TSP and asymptomatic carriers and (2) the frequency of specific CD8$^+$ T cells shows a weak positive correlation, if any, with provirus load. Furthermore, we have shown that these observations are not inconsistent with the conclusion that the median provirus load is greater in patients with HAM/TSP. The higher provirus load might result from less-efficient CTL surveillance or differences in the rate of proliferation of HTLV-1–infected cells. We conclude, first, that high detectable frequencies of HTLV-1–specific CD8$^+$ T cells implies the persistent expression of these HTLV-1 antigens in vivo and, second, that the specific CD8$^+$ T cell frequency alone cannot be used as a reliable marker either of the risk of HAM/TSP or the effectiveness of the CTL response in controlling the HTLV-1 provirus load at equilibrium.

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