High frequencies of Th1-type CD4+ T cells specific to HTLV-1 Env and Tax proteins in patients with HTLV-1–associated myelopathy/tropical spastic paraparesis


Introduction

Human T-cell lymphotropic virus type 1 (HTLV-1) was the first human retrovirus discovered.1 It is endemic in many tropical countries particularly Melanesia, the Caribbean, West Africa, Central/South America, and in Southern Japan and Iran. It is estimated to infect between 10 and 20 million people worldwide.2 Unlike human immunodeficiency virus (HIV), HTLV-1 does not cause disease in the majority of infected subjects (asymptomatic carriers [ACs]). Approximately 2% to 3% develop adult T-cell leukemia/lymphoma and another 2% to 3% develop chronic inflammatory disease of which HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP) is the most commonly recognized, but polymyositis, alveolitis, arthritis, thyroiditis, uveitis, and other end-organ inflammatory diseases have also been diagnosed.3

HTLV-1 possesses the 3 main genomic regions of env, gag, and pol (similar to other retroviruses), but unlike other leukemia viruses, it has an additional region called pX that encodes at least 2 other transcriptional regulatory proteins, the Tax and Rex proteins.3 These proteins are the homologues of the Tat and Rev proteins of HIV.4 The Rex protein stabilizes viral messenger RNAs and regulates their splicing and transport. The Tax protein is crucial to virus dynamics because as well as transactivating viral transcription, it is thought to drive host cell proliferation. The Tax protein is also the dominant target antigen recognized by HTLV-1–specific cytotoxic T lymphocytes (CTLs) in most responding individuals.5,9 So far, most investigations of the immune response to HTLV-1 have focused on the CTL response.

The CD4+ T-cell response to HTLV-1 is also important, for the following reasons: (1) CD4+ T cells are the main subset of cells infected with HTLV-1 in vivo;10,11 (2) HTLV-1–infected CD4+ T cells spontaneously secrete proinflammatory, neurotoxic cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α);12,13 and high levels of these cytokines have been demonstrated in the sera, cerebrospinal fluid (CSF), and spinal cord lesions of patients with HAM/TSP;14-16 (3) CD4+ T-cell help is required for optimal CD8+ and antibody responses;17,18 and HTLV-1 infection of CD4+ T cells may impair T-helper function;19,20 and (4) CD4+ T cells are the predominant subset of infiltrating lymphocytes detected in the central nervous system (CNS) lesions of patients with HAM/TSP in the early phase of disease.21 This observation suggests that these cells (whether they are themselves infected with HTLV-1 or not) play a role in the pathogenesis of HAM/TSP.

Efforts to study the CD4+ T-cell response to HTLV-1 in infected patients have so far been hampered by the phenomena of “spontaneous proliferation”22-24 and spontaneous cytokine production25 of peripheral blood mononuclear cells (PBMCs) after several days of culture in vitro. We have therefore developed short-term (6 hours) Elispot and intracellular cytokine detection assays to overcome these problems. We have also adopted the strategy of using overlapping peptide panels that span the entire Env and Tax proteins to examine the total CD4+ T-cell responses to all potential epitopes in these proteins, regardless of the major histocompatibility complex (MHC) class II specificities of the individual. Therefore, the aims of this study were to test the hypotheses that the frequency of HTLV-1–specific CD4+ T cells differs between patients with HAM/TSP and asymptomatic carriers, that there is a bias toward the Th1 phenotype in HTLV-1–infected subjects,
and that HTLV-1–specific CD4+ T cells are selectively infected with HTLV-1.

Our results showed significantly higher frequencies of HTLV-1 Env- and Tax–specific CD4+ T cells in patients with HAM/TSP compared to ACs. Patients with HAM/TSP also showed a significant predominance of the Th1 phenotype in these HTLV-1–specific CD4+ T cells. Furthermore, most HTLV-1–specific CD4+ T cells did not express Tax and were therefore not susceptible to lysis by HTLV-1–specific CTLs. These results have implications for the initiation or pathogenesis of HTLV-1–associated inflammatory diseases.

Patients, materials, and methods

Subjects and cells

Subjects were asymptomatic HTLV-1 carriers and patients with HAM/TSP attending the HTLV-1 clinic at St Mary’s Hospital. Infection with HTLV-1 was confirmed by the presence of antibodies to HTLV-1 gag (p19 and p24) and env (gp21 and gp46) antigens in sera by Western blot (Genelabs HTLV 2.4, Singapore). The diagnosis of HAM/TSP was made according to attending the HTLV-1 clinic at St Mary’s Hospital. Infection with HTLV-1 subjects were asymptomatic HTLV-1 carriers and patients with HAM/TSP. Subjects and cells

Flow cytometry

Cell preparation and antigen stimulation. For antigen–specific cytokine responses, 1 × 106 PBMCs depleted of CD8+ cells) were placed in 16 × 125-mm round-bottom polystyrene tissue culture tubes (Coming Costar, Cambridge, MA) with 1 mL CM, supplemented with peptides each at 1 μM final concentration and costimulatory mAbs as above. Culture tubes were incubated at 37°C in a humidified 5% CO2 atmosphere for a total of 6 hours, with the last 5 hours including a final concentration of 10 μg/mL Brefeldin A (Sigma) to inhibit secretion of cytokines from the cells. After incubation, the cells were harvested for subsequent staining.

Cell surface staining. Harvested cells were washed in PBS containing 7% normal goat serum (NGS; Sigma) and then incubated with NGS for 30 minutes at 4°C to block the Fc receptor sites on cells of the monocyte/macrophage lineage. After one wash, cells were incubated with the relevant mAbs (each at 15 μg/mL) and then washed again. Cells were then resuspended in PBS containing 7% NGS and then washed with 10 μg/mL anti–MHC class II mAb (clone 739, IgG2a; Becton Dickinson, Oxford, United Kingdom) and CD49d (clone HP2/1, Serotec), both at 0.5 μg/mL, were then added for CD4+ T-cell assays.28 Peptides were then added directly to the supernatant at a final concentration of 1 μM each and the plates incubated for 6 hours at 37°C in 5% CO2.

The plates were then incubated at room temperature for 2 hours with the second-layer biotinylated antibody to IFN-γ or IL-4 (7B6-1-biotin or IL4-II-biotin, Mabtech), both at 0.5 μg/mL. The medium was then discarded and incubated at room temperature for 1 hour. This solution was then discarded and the plates washed 6 times with PBS/0.05% Tween 20. Chromogenic alkaline phosphatase substrate (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate [NBT/BCIP], Biorad, Hertfordshire, United Kingdom) was prepared according to the manufacturer’s instructions and added at a volume of 100 μL/well. The plates were incubated for 0.5 to 1 hour at room temperature and then the reaction was terminated by washing with tap water. Plates were then allowed to air-dry and the number of spots in each well was then counted in a digital image with AID software (AID Elispot Reader, Strassberg, Germany). A response was defined as positive if the number of spots exceeded the mean ± 2 SDs of the spot count in the negative control wells (no peptides added). The frequency of cytokine-secreting CD4+ T cells was then derived by the formula: number of spots/number of CD4+ T cells per well (as ascertained previously by flow cytometry).

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Detection of intracellular Tax protein and cytokines. Cells were permeabilized with PBS/7% NGS containing 0.2% saponin (permeabilization buffer [PB]) for 10 minutes at room temperature and then washed. The cells were then resuspended in this solution with PE-labeled or fluorescein isothiocyanate (FITC)–labeled anti-IFN-γ mAb (Beckman Coulter), anti-Tax mAb (LT4), isotype controls, or mAbs to other cytokines, for example, IL-4, as appropriate for 20 minutes at room temperature. The cells were washed twice with PB and then resuspended in PB with FITC- or
PE-labeled goat F(ab')2 antiamouse IgG (Southern Biotechnology, Birmingham, AL) as appropriate for 20 minutes at room temperature. Finally, the cells were washed twice with PB, resuspended in PBS, and analyzed by flow cytometry on a Coulter EpicsXL (Beckman Coulter) with Coulter Expo 32 software.

Results

Detection of HTLV-1–specific CD4+ T cells by in vitro activation with HTLV-1 Env and Tax peptides

There has been no previous report of experimental estimates of HTLV-1–specific CD4+ T-cell frequencies in HTLV-1–infected subjects. Conventional proliferation assays cannot be used to quantify HTLV-1–specific CD4+ T-cell frequencies because of the high level of spontaneous cytokine production and proliferation in PBMCs from HTLV-1–infected people when cells are cultured in vitro. We have recently shown that HTLV-1–infected CD4+ T cells spontaneously produce detectable cytokines (initially IFN-γ) only after more than 6 hours of in vitro cultivation (no cytokines are detectable ex vivo). We have therefore devised flow cytometric and Elispot assays to directly detect HTLV-1–specific CD4+ T cells by short-term (6 hours) in vitro stimulation with Env and Tax peptides. We used peptide libraries encompassing the full-length sequences of Env and Tax to ensure that CD4+ T cells responding to all potential epitopes in these 2 proteins would be detected, regardless of their class II MHC specificity.

Figure 1A shows that HTLV-1 peptides induced detectable IFN-γ production only in CD4+ lymphocytes from the patient with HAM/TSP and not in cells from an uninfected control subject. The absence of IFN-γ+ cells in the uninfected control demonstrates the specificity of the assay. The use of an isotype control mAb also confirmed the specificity of IFN-γ staining (data not shown). This suggested that the 6-hour assay could be used for detection of HTLV-1 peptide-specific CD4+ T cells. When cultivation was extended to 12 hours or longer for samples from infected patients, the background IFN-γ staining became unacceptably high and meaningful results could not be obtained because over 10% of Tax-expressing CD4+ T cells expressed IFN-γ at 12 hours and this figure rose to about 20% at 24 hours.

Elispot assays produced an equivalent result, that is, only CD4+ T cells cultivated with HTLV-1–derived peptides for 6 hours in 96-well plates showed positive responses, defined as a spot-forming cell (SFC) count that significantly exceeded the negative controls (see “Patients, materials, and methods”).

Comparison of independent replicate Elispot assays on cells from a single blood sample, performed on different dates, showed high reproducibility (Figure 1B). We then compared data from both Elispot and intracellular cytokine staining assays on cells obtained from single blood samples. Figure 1C shows that the results were similar.

To further confirm that the peptide-induced responses were MHC class II mediated, we added anti-MHC class I and II mAbs to the culture medium to inhibit class I and II restricted responses, respectively. As expected, the responses were inhibited by anti-class II MHC antibodies but not anti-class I MHC antibodies (Figure 2).
The failure to detect IL-4 could be due to poor sensitivity of the Elispot assay used. To investigate this possibility, PBMCs depleted of CD8+ cells from all the patients were stimulated with polyclonal activators (PMA and A23187) and spots detected with our IL-4 Elispot assay. Responses ranged from 0.01% to 0.82% of CD4+ T cells (median frequency, 0.21%). This showed that a significant proportion of the CD4+ T cells retained the ability to secrete IL-4 and that these responses were detectable. The spontaneous IL-4 responses to peptides were detectable in 5 of 9 patients and ranged from 0.0020% to 0.17% of their CD4+ T cells (median, 0.10%).

High frequencies of HTLV-1 Env- and Tax-specific CD4+ T cells in HAM/TSP patients

Having established that the Th1 phenotype is predominant among HTLV-1–specific CD4+ T cells in patients with HAM/TSP; we determined the frequencies of IFN-γ–secreting HTLV-1 Env- and Tax-specific CD4+ T cells in 9 patients with HAM/TSP, 7 ACs, and 3 uninfected controls.

The results (Figure 4) show that patients with HAM/TSP had a significantly higher frequency of HTLV-1–specific CD4+ T cells than both healthy ACs and uninfected controls. These responses varied from 0.12% to 0.93% (median, 0.23%) of circulating CD4+ T cells for patients with HAM/TSP, compared to ACs in whom the frequency varied from undetectable to 0.34% (median frequency, 0.038%). Uninfected controls showed a basal level of response (median frequency, 0.035%). There was no significant difference between the responses seen in ACs and the basal level seen in uninfected controls. Experiments are now under way to define the precise peptides recognized by each individual.

Detection of Tax expression in PBMCs from HTLV-1–infected patients

We have recently devised a sensitive flow cytometric assay to detect intracellular Tax protein expression.21 We have now further characterized the sensitivity and specificity of the assay. Figure 5A shows the result of Tax staining in MT-2 cells (chronically infected with HTLV-1), Jurkat cells (uninfected), and a patient with HAM/TSP’s PBMCs (cultivated in vitro for 6 hours). Figure 5B

Figure 3. The Th1 phenotype is dominant among HTLV-1 Env- and Tax-specific CD4+ T cells in patients with HAM/TSP. This shows frequencies of IFN-γ and IL-4 SFCs/10^5 CD4+ T cells by parallel Elispot assays in 5 patients with HAM/TSP, 4 ACs, and 3 uninfected controls (Un). Statistical analyses were performed within groups using the paired Student t test. Group A: 2-tailed P = .0222 (asterisk indicates significant), IL-4 responses ranged from 0% (undetectable) to 0.14% of CD4+ T cells (median, 0.014%), IFN-γ responses ranged from 0.12% to 0.44% (median, 0.26%). Group B: 2-tailed P = .8930 (not significant), IL-4 responses ranged from 0% to 0.17% (median, 0%), IFN-γ responses ranged from 0% to 0.11% (median, .045%). Group C: 2-tailed P = .0912 (not significant), IL-4 responses were undetectable, IFN-γ responses ranged from 0.021% to 0.066% (median, 0.035%).
Figure 5. Sensitivity and specificity of intracellular Tax protein detection. (A) Dot plots showing Tax expression in Jurkat (an uninfected T-cell line) cells, MT2 cells (persistently infected with HTLV-1), and PBMCs from a patient with HAM/TSP after 6 hours of in vitro culture. (B) Dot plots showing experiments where different ratios of MT2 to Jurkat cells were mixed, stained for intracellular Tax, and analyzed by flow cytometry. All plots show 20,000 events except for the final plot, which shows 50,000 events. There are 5 cells positive for Tax seen in the final plot.

Figure 6. The majority of HTLV-1 Env- and Tax-specific CD4⁺ T cells are not infected with HTLV-1. This figure shows concomitant detection of intracellular Tax and IFN-γ expression in CD4⁺ T cells after 6 hours in vitro activation with peptides from a patient with HAM/TSP (TAF). Dot plots showed that the vast majority of Tax⁺ cells came from the IFN-γ⁺ fraction. This was representative of experiments on 5 different subjects; refer to Table 1.

shows the results of staining differing ratios of MT-2 cells with Jurkat cells. These data confirmed that the Tax staining assay was able to detect one MT-2 cell among 10,000 uninfected cells.

Proportion of HTLV-1-specific CD4⁺ T cells that express Tax protein

We wished to know whether HTLV-1-specific CD4⁺ T cells were selectively infected with HTLV-1 because infection has been shown to alter the function of T-helper cells.¹⁹,²⁰ Recent data from our laboratory suggested that there is preferential infection of HTLV-1-specific CTLs.³⁰ Therefore, staining for intracellular Tax expression was combined with the 6-hour flow cytometric assay for intracellular IFN-γ. One representative experiment from 5 subjects is shown in Figure 6.

Table 1 shows that Tax expression was less frequent in HTLV-1-specific CD4⁺ T cells than CD4⁺ T cells of other specificities, in 2 patients with HAM/TSP, whereas Tax expression was more frequent in HTLV-1-specific CD4⁺ T cells in both ACs and a third patient with HAM/TSP. This contrasts with our previous finding that Tax expression in CD8⁺ T cells was detected only among HTLV-1-specific cells (in 5 of 5 individuals) and not among Epstein-Barr virus (EBV)–specific cells (0 of 4 individuals).²⁰

The fact that most HTLV-1-specific CD4⁺ T cells were not infected with transcriptionally active virus, also implies that they were not susceptible to CTL-mediated killing in vivo. Thus, the majority of circulating HTLV-1-specific CD4⁺ T cells remain capable of producing proinflammatory cytokines on encountering their cognate antigen.
Table 1. Detection of Tax protein expression in CD4+ T cells from HTLV-1-infected subjects

<table>
<thead>
<tr>
<th>Percentage of Tax-expressing cells</th>
<th>IFN-γ− cell</th>
<th>IFN-γ+ cell</th>
</tr>
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<tbody>
<tr>
<td>TAF</td>
<td>3.3</td>
<td>5.8</td>
</tr>
<tr>
<td>TAT</td>
<td>0.4</td>
<td>6.1</td>
</tr>
<tr>
<td>TW</td>
<td>1.7</td>
<td>0.6</td>
</tr>
<tr>
<td>HAY</td>
<td>10.7</td>
<td>0.4</td>
</tr>
<tr>
<td>HT</td>
<td>5.9</td>
<td>0.8</td>
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The 6-hour flow cytometric assay for intracellular IFN-γ was combined with staining for Tax protein expression. Patients with HAM/TSP have codes beginning with T and ACs have codes beginning with H.

These data do not exclude the possibility that some infected HTLV-1–specific CD4+ cells are unable to produce cytokines when activated with their respective antigen and thus escape detection in these assays. Testing of this point may have to await the advent of MHC class II tetramer or similar technology.

Discussion

The role of the cellular immune response in HTLV-1 infection is not fully understood. To date, the focus has been on the CTL response to the virus and there are few published data on the CD4+ T-cell response to HTLV-1. However, the importance of the CD4+ T-cell response should not be underestimated, because it has been shown to be essential for efficient humoral and cellular immune responses, including the CTL response. The situation is complicated further by the fact that the main subset of lymphocytes infected with HTLV-1 in vivo is the effector/memory CD4+ T cell. The paucity of data on the HTLV-1–specific CD4+ T-cell response reflects the problems encountered with the phenomena of spontaneous lymphocyte proliferation and cytokine production seen in PBMCs from these infected patients.

Using short (6-hour) cultures we have eliminated the effect of spontaneous proliferation of lymphocytes from our assays. This has enabled us to demonstrate HTLV-1–peptide–specific CD4+ T-cell responses in subjects infected with HTLV-1. We have found that these cells exhibit a Th1 phenotype and we therefore postulate a role for these cells in the pathogenesis of HTLV-1–associated inflammatory disease, especially in HAM/TSP. Pathologic examination of the spinal cord has shown that in patients with HAM/TSP with a short duration of symptoms, the characteristic perivascular lymphocyte infiltrate consists predominantly of CD4+ T cells; only later do CD8+ T cells predominate, and finally an atrophic picture is seen.

Whether the CD4+ T cells present in HAM/TSP lesions are predominantly HTLV-1 infected or HTLV-1 specific has not been shown. However, if HTLV-1–specific CD4+ T cells of the Th1 phenotype are also frequent among the CD4+ T cells that infiltrate CNS lesions (as they are in peripheral blood, which we show in this paper), then such cells may be important in the pathogenesis of HAM/TSP, particularly in early lesions, through the secretion of neurotoxic cytokines such as IFN-γ. A similar role has been suggested for HTLV-1–specific CTLs, which predominate later in disease. However, relatively little is known of the functions of the infiltrating lymphocytes; both cell types may contribute to pathogenesis, in varying proportions, throughout the course of the disease.

This observation that HTLV-1–specific CD4+ T cells in HAM/TSP patients are mainly of the Th1 phenotype is important because a dominant Th1 response could be either beneficial, harmful, or both. A strong Th1 response could be beneficial because it is associated with a strong antiviral CTL response and therefore with effective control of HTLV-1 infection, or harmful because the production of proinflammatory cytokines could lead to “bystander damage” to uninfected cells. It is possible that the balance of good and harm done by HTLV-1–specific CD4+ T cells depends on factors such as the proviral load, in a manner analogous to the mechanism we have proposed in the CD8+ T-cell response to HTLV-1.

Furthermore, we have shown that the frequencies of HTLV-1 Env- and Tax-specific CD4+ T cells are significantly greater in HAM/TSP patients than in ACs. Activated Th1 cells (but not Th2) are reported to have a strong tendency to cross vessel walls (including the blood-brain barrier) and infiltrate areas of inflammation. Therefore, we suggest that these abundant Th1-type HTLV-1–specific CD4+ T cells are likely to encounter cognate antigens in the tissues (eg, the CNS) when the proviral load is high (as in patients with HAM/TSP), because the frequencies of both the Th1 CD4+ T cells and infected CD4+ T cells are high. The high frequencies of specific CD4+ T cells suggest that there is continuous antigen stimulation in vivo, implying in turn that the virus is not latent.

It is well established that HTLV-1 infection is associated with a high proviral load in peripheral blood. It is also known that the main burden of HTLV-1 infection in vivo is carried by the activated memory subset of CD4+ T cell (CD3+, CD4+, CD45RO+). Furthermore, we have previously shown that a majority of these infected cells were clustered in the effector/memory subpopulation (CCR7−, CD62L−). Therefore, it was important to know whether the majority of the HTLV-1–specific CD4+ T cells were infected with HTLV-1. Recent work from this laboratory suggested that HTLV-1–specific CD8+ CTLs were preferentially infected by HTLV-1, whereas EBV-specific CD8+ CTLs were not.

Infection of HTLV-1–specific CD4+ T cells would have the following implications: (1) disruption of normal in vivo function by up-regulation and transactivation of cellular gene expression by Tax, and (2) viral protein expression in infected T cells would render them susceptible to killing by autologous CTLs. Taken together, these could seriously impair or weaken the CD4+ T-cell immune response to HTLV-1. Using our newly devised sensitive and specific flow cytometric assay, we have shown that the majority of these HTLV-1 Env- and Tax-specific CD4+ T cells did not express the Tax protein after 6 hours of culture in vitro. This observation suggested that most HTLV-1–specific CD4+ T cells were not infected by transcriptionally active HTLV-1 and therefore remained capable of normal cytokine production on encountering antigen.

In conclusion, we demonstrate significantly higher frequencies of Env- and Tax-specific CD4+ T cells in patients with HAM/TSP than in ACs. We also show that the Th1 phenotype is dominant among these cells in patients with HAM/TSP. The majority of these Th1-type HTLV-1–specific CD4+ T cells are also shown not to express Tax protein after short in vitro culture and thus remain capable of responding normally when encountering cognate antigen. We suggest that these cells are involved in the initiation and pathogenesis of inflammatory disease in patients with HAM/TSP by the production of proinflammatory cytokines when activated by HTLV-1 antigens in the CNS and other end organs.

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References


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