Brief Definitive Report

Evidence for the Chronic In Vivo Production of Human T Cell Leukemia Virus Type I Rof and Tof Proteins from Cytotoxic T Lymphocytes Directed against Viral Peptides

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Abstract

Human T cell leukemia virus type I (HTLV-I) is a persistent virus that causes adult T cell leukemia and tropical spastic paraparesis/HTLV-I–associated myelopathy. Studies on rabbits have shown that viral proteins encoded by the open reading frames pX-I and pX-II are required for the establishment of the persistent infection. To examine the in vivo production of these proteins in humans, we have investigated whether cytotoxic T lymphocytes isolated from HTLV-I–infected individuals recognized pX-I and pX-II peptides. $CD8^+$ T lymphocytes to pX-I and pX-II peptides were detected in HTLV-I–infected individuals, whatever their clinical status, and even in the absence of any antigenic restimulation. These findings indicate that the HTLV-I pX-I and pX-II proteins are chronically synthesized in vivo, and are targets of the natural immune response to the virus.

Key words: retrovirus • regulatory proteins • cytotoxic epitopes • HLA-A2 • interferon γ

Introduction

Human T cell leukemia virus type I (HTLV-I) is the etiological agent responsible for adult T cell leukemia (ATL) and the tropical spastic paraparesis/HTLV-I–associated myelopathy (TSP/HAM). HTLV-I is a complex retrovirus. The 3′ region of its genome (termed pX) encodes distinct regulatory proteins in addition to the structural genes common to most retroviruses. Open reading frames (ORFs) pX-III and pX-IV encode the posttranscriptional regulator Rex protein and the viral transactivator Tax protein, respectively, which have been extensively studied (1). In contrast, ORFs pX-I and pX-II encode proteins whose functions in the viral cycle have not yet been elucidated. These proteins are each produced from single- and double-spliced transcripts (2). The double-spliced pX-I and pX-II transcripts encode the Rof and Tof proteins, respectively, whereas the single-spliced pX-I and pX-II RNAs encode the $p12^I$ protein consisting of the last 98 residues of Rof, and the $p13^{II}$ protein corresponding to the last 87 residues of Tof, respectively (see Fig. 1 A). After transfection, both the Tof and $p13^{II}$ proteins are produced from their respective pX-II cDNAs, whereas only the $p12^I$ protein is produced from both the double- and single-spliced pX-I RNAs (2).

Neither pX-I nor pX-II protein is required for virus replication in vitro (3). However, both are important in vivo, since the HTLV-I $p12^I$ protein and the Tof protein of HTLV-II are required for the establishment of a persistent infection in rabbits (4, 5). HTLV-I $p12^I$ and Tof proteins probably play a similarly critical role in human infection, but their production in HTLV-I–infected individuals remains to be proven.

Proteins encoded by pX-I and pX-II ORFs are not, or are very poorly, recognized by sera from HTLV-I–infected individuals (6). Therefore, to examine the in vivo production of these proteins, we have investigated whether they are targets of the cytotoxic T cell response generated during HTLV-I infection. We established cytotoxic T cell lines from HLA-A2 HTLV-I–infected individuals with various clinical status, and studied their ability to recognize pX-I

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and pX-II peptides. We also examined whether $CD8^+$ effectors directed to pX-I and pX-II peptides were chronically generated during HTLV-I infection.

Materials and Methods

Subjects. HTLV-I–infected patients and control donors were selected on the basis of HLA-A2 molecule expression. All patients gave their informed consent. Blood samples were collected from five HTLV-I–positive asymptomatic carriers (41948, 44669, 34522, 15610, and 34672) and three TSP/HAM patients (COU, MAD, and GUI), originating from the French West Indies, and from one ATL patient (ED) originating from French Guyana. HTLV-I seropositivity was verified by the presence of anti–HTLV-I antibodies by ELISA and by Western blot.

HTLV-I–uninfected donors 45542 and 34345 were recruited in the French West Indies, and uninfected donors 821, 72, and 817 in metropolitan France.

Peptides. The HLA-A2–restricted CTL epitopes, peptide 27–35 of the melanoma-associated protein Mart-1 (7; provided by F. Faure, Institut National de la Santé et de la Recherche Médicale, U520, Institut Curie, Paris) and peptide 77–85 of the HIV-GAG protein (8; donated by F. Lemonnier, Institut Pasteur, Paris), were used as negative controls. The 9-mer pX-I and pX-II peptides used in this study (located downstream of the regions shared with Rex and Tax, as shown in Fig. 1, B and C) have been described previously (9). Tax, pX-I, and pX-II peptides were synthesized with the PepSet synthesis system (Chiron Mimotopes), suspended in water at 2 mM, and stored at −20°C.

Cell Lines. B lymphoblastoid cell lines (B cell lines) were established by immortalizing peripheral B lymphocytes with EBV.

Polyclonal CTL lines were obtained from unfrozen PBMCs, and cultured for 3 d in RPMI medium supplemented with 5% FCS and 5% human serum (Sigma Chemical Co.). $CD8^+$ T cells were then isolated by positive selection using anti-CD8–coated magnetic microbeads (MACS reagent; Tebu), suspended in 2 ml culture medium, and stimulated with 1 μg/ml phytohemagglutinin (PHA-M; Sigma Chemical Co.) plus 50 U/ml IL-2 (Roussel Uclaf).

Chromium-Release Cytotoxic Assay. 5×10^5 cells from the different B cell lines were incubated with 100 μCi ^{51}Cr (NEN) in 100 μl culture medium, with or without peptides (5 μM), for 1 h at 37°C, washed twice with culture medium, and plated in duplicate in a 96-well (round-bottomed) culture plate (2,000 cells per well in 100 μl of culture medium). $CD8^+$ T cells were then added into 100 μl culture medium at an E/T ratio of 40:1, and the plates were incubated at 37°C for 5 h. In some cases, anti-HLA class I antibody (W6/32, 20 μg/ml; Sigma Chemical Co.) and anti-HLA class II antibody (TU-36, 20 μg/ml; Tebu) were added to the culture medium for the 5-h period. Aliquots (100 μl) of supernatant were collected, and the radioactivity, corresponding to released chromium, was counted in a gamma counter (Amersham Pharmacia Biotech).

ELISPOT Assay. Nitrocellulose plates (96-well; Millipore) were coated with 50 μl anti–human IFN-γ mAb (2 μg/ml; Genzyme Corp.) in carbonate buffer (15 mM $NaHCO_3$, 33 mM Na_2CO_3, pH 9.5) for 16 h at 4°C. The wells were washed twice with culture medium and incubated with 200 μl of the same medium for 2 h at 37°C. Purified $CD8^+$ T lymphocytes were then plated in triplicate at 5×10^4/well, and peptides (5 μM) were added in a total volume of 200 μl. As a positive control, 500 $CD8^+$ cells were also activated with phorbol ester (PMA, 50 ng/ml; Sigma Chemical Co.) and ionomycin (500 ng/ml; Sigma Chemical Co.). The plates were incubated for 24 h at 37°C, then washed six times with PBS containing 0.05% Tween 20 (PBS-Tween). Polyclonal rabbit anti–human IFN-γ (100 μl of a 1:250 dilution in PBS-Tween containing 1% BSA; Genzyme Corp.) was added to each well, and the plates were incubated at 4°C overnight. They were washed six times with PBS-Tween, and biotin-conjugated anti–rabbit $F(ab')_2$ fragment (100 μl of a 1:500 dilution in PBS-Tween-BSA; Boehringer Mannheim) was added to each well. The plates were incubated for 1 h at 37°C, washed six times, and incubated with alkaline phosphatase–conjugated streptavidin (100 μl of a 1:6,000 dilution in PBS-Tween-BSA; Sigma Chemical Co.) for 1 h at 37°C. Plates were washed six times, 100 μl alkaline phosphatase substrate (Bio-Rad) was added to each well, and the plates were then incubated for 1–2 h. Finally, the plates were washed in water and air-dried, and the colored spots were counted under a stereomicroscope.

Results

Recognition of pX-I and pX-II Peptides by CTLs Expanded from HLA-A2 TSP/HAM Patients. We showed previously that one peptide located in the pX-I protein (Rof 57–65; Fig. 1 B) and another in the pX-II protein (Tof 156–164; Fig. 1 C) were able to associate to HLA-A2 molecules in vitro (9). We have now examined whether CTLs directed

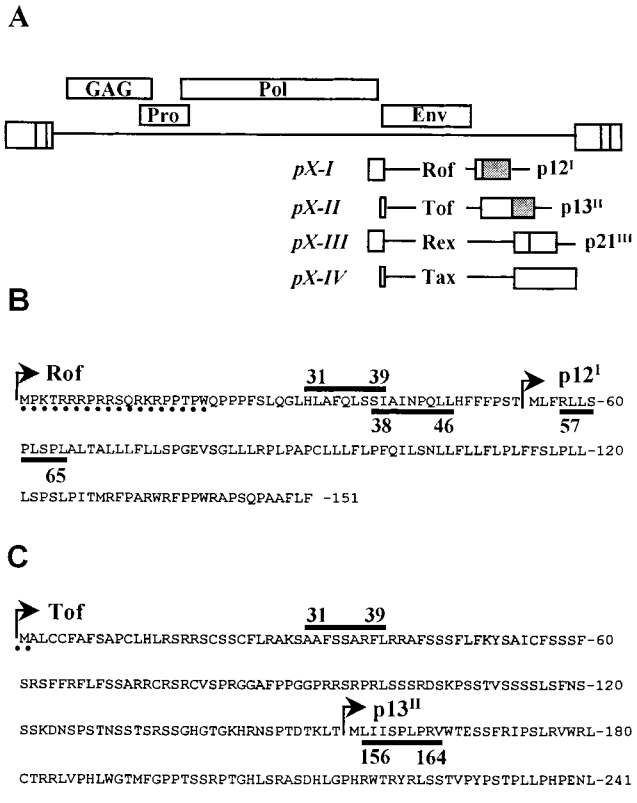

Figure 1. Diagram showing the HTLV-I genome and the locations of the pX-I and pX-II peptides. (A) The structural (top) and regulatory (bottom) genes of HTLV-I. The coding sequences of the truncated proteins $p12^I$ and $p13^{II}$ are shaded. (B) The pX-I peptides in the amino acid sequences of Rof and $p12^I$ proteins. The Rof and $p12^I$ initiator codons are indicated by arrows, and the residues shared by the Rex and Rof proteins are dotted. (C) The pX-II peptides in the amino acid sequences of the Tof and $p13^{II}$ proteins. The Tof and $p13^{II}$ initiator codons are indicated by arrows, and the first residue shared by the Tax and Tof proteins is dotted.

against these peptides were detected in HLA-A2 HTLV-I–infected individuals.

We first tested samples from TSP/HAM patients known to have a greater CTL response to HTLV-I than asymptomatic carriers (10). We also cultivated PBMCs for 3 d before expansion, to allow restimulation of anti–HTLV-I $CD8^+$ T lymphocytes by autologous $CD4^+$ infected T cells, and so maximize the frequency of antiviral CTLs. The cytotoxic activities of polyclonal $CD8^+$ lines were then tested against peptide-loaded HLA-A2–autologous B cell lines after 10–15 d in culture.

There was no cell lysis when CTL lines from the TSP/HAM patients were incubated with autologous target cells with no peptide, or pulsed with the control HLA-A2–restricted CTL epitopes Mart-1 27–35 or HIV-GAG 77–85 (<5% cell lysis; Fig. 2 A). In contrast, each CTL line lysed target cells pulsed with the 11–19 peptide (48–82% cell lysis) located in the Tax protein, which is the immunodominant target of the anti–HTLV-I CTL response (11). CTL lines from the TSP/HAM patients also lysed target cells pulsed with the Rof 57–65 peptide (14–23% cell lysis) or with the Tof 156–164 peptide (22–34% cell lysis) (Fig. 2 A). The Tof 156–164 peptide caused cell lysis up to a concentration of 100 nM, whereas recognition of the Rof 57–65 peptide required higher concentrations (Fig. 2 B), which is consistent with the relative capacities of these peptides to associate with HLA-A2 molecules in vitro (moderate and low binder peptides, respectively; reference 9). The recognition of the Rof 57–65 and Tof 156–164 peptides was inhibited by adding anti-HLA class I, but not anti-HLA class II, antibodies (Fig. 2 C). Finally, target cell lysis was observed when the peptides were loaded on various HLA-A2–matched target cells, but not on non–HLA-A2 cells (data not shown). The generation of CTLs to pX-I and pX-II peptides was not a characteristic of TSP/HAM patients, since the Rof 57–65 and Tof 156–164 peptides were also recognized by CTL lines established from three HLA-A2–asymptomatic carriers (41948, 44669, and 34672) and one HLA-A2 ATL patient (ED) (Rof 57–65, 12–32% cell lysis; and Tof 156–164, 12–38% cell lysis; Fig. 2 D). In contrast, there was no antipeptide CTL activity in three CTL lines from HLA-A2–uninfected donors (Fig. 2 E). That CTLs to pX-I and pX-II peptides were generated in HTLV-I–infected individuals, whatever their clinical status, and not in uninfected donors, strongly suggests that the corresponding proteins are produced in vivo and that this production is a common feature of HTLV-I infection.

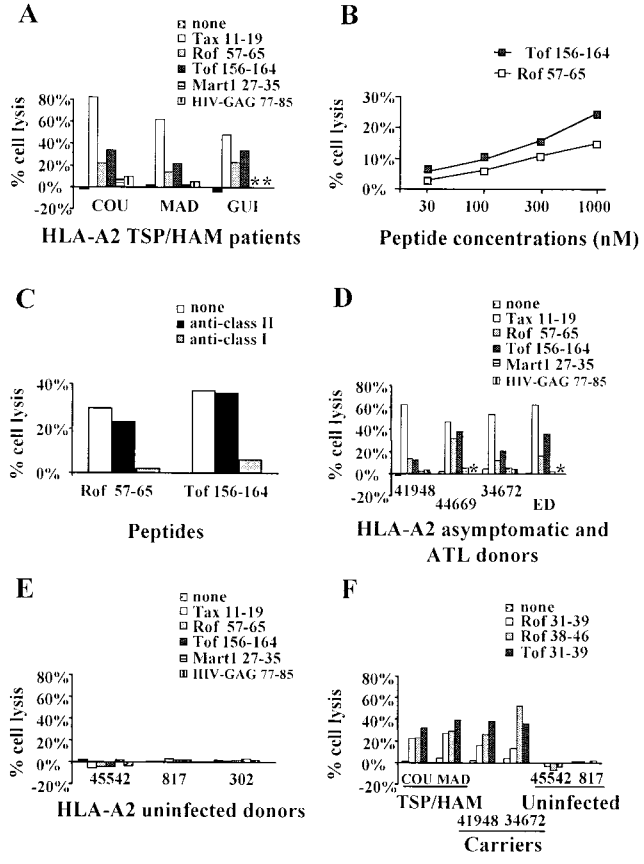

Figure 2. Cytotoxic activity of polyclonal CTL lines detected in the chromium-release assay. (A) Reactivity of CTL lines established from HLA-A2 TSP/HAM patients against Tax, pX-I, pX-II, and control HLA-A2–restricted CTL epitopes. (B) Determination of the concentrations of Rof 57–65 and Tof 156–164 peptides required for recognition by the CTL line from a TSP/HAM patient. (C) Effect of anti-HLA class I and class II antibodies on recognition of the Rof 57–65 and Tof 156–164 peptides by the CTL line from a TSP/HAM patient. (D) Reactivity of CTL lines established from HLA-A2 HTLV-I carriers and one ATL patient (ED) or (E) HLA-A2–uninfected controls, against Tax, pX-I, pX-II, and control peptides. (F) Reactivity of CTL lines established from HLA-A2 TSP/HAM patients, HLA-A2–infected carriers, and HLA-A2–uninfected controls against Rof and Tof peptides. *Peptides not tested.

CTLs from HTLV-I–infected Patients Recognized Peptides within the Full-Length Rof and Tof Proteins. We next tested whether CTLs from HTLV-I–infected patients recognized peptides produced only from the full-length pX-I and pX-II proteins to determine whether the complete Rof and Tof proteins were synthesized in vivo. We used the Rof 31–39 peptide (Fig. 1 B) previously shown by us to bind moderately to HLA-A2 molecules (9). We also used the Rof 38–46 and Tof 31–39 peptides (Fig. 1, B and C) previously considered by us to be HLA-A2–nonbinder peptides because they associated with HLA-A2 molecules only at high concentrations (our unpublished results). Since examples of peptides that associate weakly with HLA molecules in vitro but are potent CTL epitopes have been reported (12), these peptides were tested despite their poor binding. CTL lines from two HLA-A2 TSP/HAM patients and two HLA-A2 HTLV-I carriers recognized the Rof 31–39 peptide (13–27% cell lysis), the Rof 38–46 peptide (23–53% cell lysis), and the Tof 31–39 peptide (32–39% cell lysis), whereas there was no activity in two HLA-A2–uninfected donors (<2% cell lysis) (Fig. 2 F). As for the Rof 31–39 and Tof 156–164 peptides, the Rof 31–39, Rof 38–46, and Tof 31–39 peptides were only presented by HLA-A2 target cells, and their recognition was inhibited by anti-HLA class I antibodies (data not shown). Hence, we identified HLA-A2–restricted CTL epitopes produced only from the full-length Rof and Tof proteins. This indicates that the Rof protein, although not detected in vitro, is well synthesized

in vivo, and that the Tof protein, detected in vitro, is also produced in vivo.

In Vivo–primed CTLs against pX-I and pX-II Peptides Are Present in HLA-A2 HTLV-I Carriers. Finally, we determined whether $CD8^+$ effectors directed against Rof and Tof peptides were detected without any restimulation of $CD8^+$ T lymphocytes by viral products. Since immune effectors are short-lived cells (13), such a result would imply that some $CD8^+$ T lymphocytes had been primed in vivo from Rof and Tof proteins in current production.

We first tried to detect an ex vivo anti–HTLV-I cytotoxic activity using the chromium-release assay and found no response to the Rof and Tof peptides, even though there was a slight response to the Tax 11–19 peptide (data not shown). We next used the IFN-γ enzyme-linked immunospot (ELISPOT) assay (14), which is much more sensitive than the chromium-release assay, to increase the probability of detecting ex vivo activity to HTLV-I peptides. Since it has been shown that $CD4^+$ T lymphocytes from TSP/HAM patients secrete high levels of IFN-γ (15), we used purified $CD8^+$ T lymphocytes instead of PBMCs. However, there were many spots in the absence of peptide stimulation, even with purified $CD8^+$ T lymphocytes from TSP/HAM blood samples (data not shown), which rendered the interpretation of the results difficult. Assuming that this background was due to the general immune activation characteristic of TSP/HAM patients (16), we repeated this experiment using samples from HTLV-I carriers. Table I shows the results obtained with freshly purified $CD8^+$ T cells from three HLA-A2 HTLV-I carriers and three HLA-A2–uninfected donors as negative controls. No responses against Tax, pX-I, pX-II, or control peptides were detected in samples from uninfected donors (<60 spots per 10^6 $CD8^+$ cells), although they produced large amounts of IFN-γ in response to mitogens. In contrast, there were many spots (200–2,180) in samples from the three HTLV-I carriers in response to the immunodominant Tax 11–19 peptide. Two HTLV-I carriers (34522 and 15610) also responded to the Tof 31–39 peptide (660 and 340 spots, respectively) and to the Tof 156–164 peptide (360 and 260 spots, respectively). All of the HTLV-I carriers recognized peptides from the Rof protein, since the Rof 38–46 peptide was recognized by two carriers (15610 and 34522; 180 and 500 spots, respectively), and the Rof 31–39 peptide was recognized by the third (34672, 140 spots). Finally, none of the $CD8^+$ population isolated from the HTLV-I carriers responded to the control HLA-A2–restricted CTL epitopes (<80 spots).

The presence of circulating $CD8^+$ effectors against Rof peptides in blood samples from the three HTLV-I carriers strongly suggests that the full-length Rof protein is chronically produced in vivo. That two out of three samples from HTLV-I carriers respond to the Tof peptides also suggests that the Tof protein is constantly synthesized during HTLV-I infection.

Discussion

We have found that HLA-A2–restricted CTLs against HTLV-I pX-I and pX-II peptides are present in HTLV-I–infected individuals, regardless of their clinical status, while no such activity was present in HLA-matched uninfected controls. We have also demonstrated that some of these effectors have been primed by pX-I and pX-II peptides that are constantly produced in vivo. Finally, we have identified three HLA-A2–restricted epitopes in the pX-I ORF (Rof 31–38, Rof 38–46, and Rof 57–65) and two in

Table I. *Detection of In Vivo–primed IFN-γ–producing $CD8^+$ Effectors Directed to Tax, Rof, and Tof Peptides in HLA-A2 HTLV-I–infected Asymptomatic Carriers*

	HTLV-I–infected carriers					Uninfected donors				
	15610	34672	34522	Positivity	Mean frequency*	45542	34345	821	Positivity	Mean frequency
Mitogen	46,000	112,000	134,000	3/3	1/10	66,000	40,000	40,000	3/3	1/20
Control peptides										
Mart-1 27–35	0	80	−20	0/3	1/50,000	17	60	20	0/3	1/33,000
HIV-GAG 77–85	20	0	NT	0/2	1/100,000	NT	20	20	0/2	1/50,000
HTLV-I peptides										
Tax 11–19	500	200	2,180	3/3	1/1,000	17	40	0	0/3	1/50,000
Rof 31–39	0	140	80	1/3	1/14,000	17	40	0	0/3	1/50,000
Rof 38–46	180	60	500	2/3	1/4,000	0	40	0	0/3	1/75,000
Tof 31–39	340	0	660	2/3	1/3,000	0	40	−40	0/3	0
Tof 156–164	260	80	360	2/3	1/4,000	0	40	0	0/3	1/75,000

Data shown are the number of IFN-γ–producing cells per 10^6 fresh $CD8^+$ T lymphocytes, and are means of triplicates of a representative experiment out of two. The values represent the number of spots after subtraction of the background observed in the absence of peptide. Peptides inducing a production of IFN-γ corresponding to >100 spots/10^6 $CD8^+$ T lymphocytes were considered as positive. NT, not tested.
*Calculated by dividing 10^6 by the mean number of spots.

the pX-II ORF (Tof 31–39 and Tof 156–164), some of which are located in the complete Rof and Tof proteins. Since it seems very unlikely that these peptides are synthesized without the translation of their respective ORFs, our results strongly suggest that the Rof and Tof proteins are both chronically produced during HTLV-I infection.

The CTL response to the Tof 156–164, Rof 31–38, and Rof 57–65 peptides is in good agreement with our previous finding that these peptides bind to HLA-A2 molecules in vitro (9). We also found that the Rof 38–46 and the Tof 31–39 peptides act as HLA-A2–restricted epitopes, although they were not previously considered as HLA-A2 binders. Nevertheless, these two peptides can stimulate $CD8^+$ T lymphocytes from HTLV-I–infected donors to kill HLA-A2 target cells and to produce IFN-γ, and their recognition was inhibited by anti-HLA class I antibodies. Therefore, our results confirm that there are some notable exceptions to the general rule that a direct relationship exists between the affinity of peptides for HLA molecules in vitro and their abilities to act as CTL epitopes in vivo, as reported previously by others (12).

The production of pX-I and pX-II proteins in all HTLV-I–infected individuals is consistent with their roles in the establishment of persistent infection in vivo. Our results suggesting that the pX-I proteins are chronically produced in vivo, at least in HTLV-I carriers, may also imply that their functions are required throughout the viral infection. It has been recently reported that the pX-I $p12^I$ protein can associate with MHC class I molecules and cause their intracellular degradation, which may prevent antigenic presentation of viral proteins by infected cells (17). Constant production of the $p12^I$ protein in vivo would ensure their escape from the immune cytotoxic response.

The Rof and Tof proteins are not, or are very poorly, recognized by sera from HTLV-I–infected individuals (2). Hence, it seems that these proteins cannot induce an antibody response. This inability could be due to their location in intracellular compartments preventing them from being exposed to the extracellular environment. However, the identification of CTL epitopes in the Rof and Tof proteins demonstrates that these proteins are available for the MHC class I presentation pathway, and that they are targets of the natural anti–HTLV-I immune response.

Indeed, we found that CTLs directed against Rof and Tof peptides are present in TSP/HAM patients and in asymptomatic carriers. Moreover, the result obtained with patient ED suggests that ATL patients also generate anti-Rof and anti-Tof CTLs. Therefore, our results indicate that the antiviral CTL response is diversified toward several regulatory proteins, not only to the Tax protein, in all HTLV-I–infected individuals. That anti-Rof and anti-Tof CTLs were generated in one ATL donor is especially interesting since viral expression is believed to be very low in these patients. Nevertheless, our results suggest that sufficient levels of the Rof and Tof proteins are produced in leukemic patients to be targeted by the immune response.

The frequencies of in vivo–primed $CD8^+$ effectors against Rof 38–46, Tof 31–39, and Tof 156–164 peptides were between 1/4,000 and 1/3,000. These frequencies are in the same range as those obtained in HIV-1–positive individuals after stimulation with relevant peptides (14). The frequency of effectors against the Tax 11–19 peptide (1/1,000) was much lower than those previously reported in TSP/HAM patients using HLA-A2/Tax 11–19 tetramers (up to 10% PBMCs; reference 18). This difference is not surprising, since only in vivo–primed cytotoxic effectors were detected under our conditions, while tetramers detect all anti-Tax 11–19 CTLs, and since carriers had a lower anti-Tax CTL response than TSP/HAM patients (10). Therefore, our results confirm that HTLV-I carriers have in vivo–primed $CD8^+$ effectors to Tax (19), and demonstrate that they also possess in vivo–preactivated effectors to Rof and Tof peptides. We could not assess the frequency of in vivo–primed anti-Rof and anti-Tof $CD8^+$ effectors in samples from TSP/HAM patients because they produced a high basal amount of IFN-γ. Therefore, it remains to be determined whether the intensity of the anti-Rof and anti-Tof cytotoxic response plays a role in the pathogenesis of the TSP/HAM disease. However, despite minimization by the high background, a response to the Tax 11–19 peptide was detectable in these patients (data not shown), which confirms that they chronically generated IFN-γ–producing $CD8^+$ lymphocytes to the Tax protein, as reported recently by others (20).

This study establishes that CTLs against Rof and Tof proteins are generated during HTLV-I infection. The next question is whether these CTLs can kill HTLV-I–infected cells. This point could not be addressed in this study since it requires the production of CTL lines or clones specific for each Rof and Tof peptide. Therefore, further experiments are needed to determine whether the cytotoxic response to Rof and Tof proteins could destroy infected cells in vivo and hence, play a role in the control of the HTLV-I infection. Nevertheless, the immunological approach presented here provided evidence for the in vivo chronic production of the HTLV-I Rof and Tof proteins. This approach could be extended to other persistent infections in which viral products are, as in HTLV-I infection, physically undetectable.

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