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 from HLA-A2+ individuals recognized pX-I and pX-II peptides and 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 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 p12I and p13II proteins consisting of the last 98 residues of Rof and the last 87 residues of Tof, respectively (see Fig. 1 A). After transfection, both the p12I and p13II proteins are produced from their respective pX-I and pX-II cDNAs, whereas only the p12I 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 p12I protein and the Tof protein of HTLV-II are required for the establishment of a persistent infection in rabbits (4, 5). HTLV-I p12I and Tof proteins probably play a similar 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 and pX-II proteins corresponding to the last 87 residues of Tof, respectively (see Fig. 1 A). After transfection, both the Tof and p13II proteins are produced from their respective pX-II cDNAs, whereas only the p12I protein is produced from both the double- and single-spliced pX-I RNAs (2).

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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 M art-1 (7; provided by F. Faure, Institut National de la Santé et de la Recherche Médicale, U 520, 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), and 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.

Polycional 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 incubated at 37°C for 1 h. In some cases, anti-CD14 antibody (W6/32, 20 ng/ml; Sigma Chemical Co.) 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 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 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; 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 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 Expans were obtained from HTLV-I–positive asymptomatic carriers (41948, 44669, 34522; COU, MAD, and GUI), originating from the French West Indies, and from one ATL patient (ED) originating from French Guyana. Polycional 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.

**A**

| GAG | Pro | Pel |
|-----|-----|-----|
|     |     |     |

**B**

| pX-I | pX-II | pX-III | pX-IV |
|------|-------|--------|-------|
|       |       |       |       |

**C**

| pX-I | pX-II | pX-III | pX-IV |
|------|-------|--------|-------|
|       |       |       |       |

**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. T The coding sequences of the truncated proteins p12 and p13 are shaded. (B) The pX-I peptides in the amino acid sequences of the Rof and p12 proteins. The Rof and p12 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 proteins. The Tof and p13 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+ T cell 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 R of 57–65 peptide (14–23% cell lysis) or with the Tof of 156–164 peptide (22–34% cell lysis) (Fig. 2 A). The T of 156–164 peptide caused cell lysis up to a concentration of 100 nM, whereas recognition of the R of 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 R of 57–65 and T of 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 R of 57–65 and T of 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) (R of 57–65, 12–32% cell lysis; and T of 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.

CTCs from HTLV-I-infected Patients Recognize 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 R of and T of proteins were synthesized in vivo. We used the R of 31–39 peptide (Fig. 1 B) previously shown by us to bind moderately to HLA-A2 molecules (9). We also used the R of 38–46 and T of 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 R of 31–39 peptide (13–27% cell lysis), the R of 38–46 peptide (23–53% cell lysis), and the T of 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 R of 31–39 and T of 156–164 peptides, the R of 31–39, R of 38–46, and T of 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 R of and T of proteins. This indicates that the R of protein, although not detected in vitro, is well synthesized.

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 R of 57–65 and T of 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 R of 57–65 and T of 156–164 peptides by the CTL line from a TSP/HAM patient. (D) Reactivity of CTL lines established from HLA-A2 HTLV-I patients 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 R of and T of peptides. Peptides not tested.
in vivo, and that the T of 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 R of T 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 R of 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 T of 31–39 peptide (660 and 340 spots, respectively) and to the T of 156–164 peptide (360 and 260 spots, respectively). All of the HTLV-I carriers recognized peptides from the R of protein, since the R of 38–46 peptide was recognized by two carriers (15610 and 34522; 180 and 500 spots, respectively), and the R of 31–39 peptide was recognized by the third (34762, 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 R of peptides in blood samples from the three HTLV-I carriers strongly suggests that the full-length R of protein is chronically produced in vivo. That two out of three samples from HTLV-I carriers respond to the T of peptides also suggests that the T of protein is constantly synthesized during HTLV-I infection.

**Table I.** Detection of In Vivo–primed IFN-γ Producing CD8+ Effectors Directed to Tax, R of, and T of Peptides in HLA-A2 HTLV-I–infected Asymptomatic Carriers

| HLA-A2 HTLV-I–infected carriers | Uninfected donors |
|---------------------------------|------------------|
| HTLV-I peptides                 | Mean frequency*  |
| T of 31–39 500                  | 1/1,000          |
| R of 31–39 0                    | 1/1,000          |
| T of 156–164 260                | 1/4,000          |

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 R of 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 R of and T of proteins are both chronically produced during HTLV-I infection.

The CTL response to the T of 156–164, R of 31–38, and R of 37–65 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 R of 38–46 and the T of 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 p121 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 p121 protein in vivo would ensure their escape from the immune cytotoxic response.

The R of and T of 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 immune 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 R of and T of 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 R of and T of 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-R of and anti-T of 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-R of and anti-T of 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 R of and T of proteins are produced in leukemic patients to be targeted by the immune response.

The frequencies of in vivo–primed CD8+ effectors against R of 38–46, T of 31–39, and T of 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 R of and T of peptides. We could not assess the frequency of in vivo–primed anti-R of and anti-T of 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-R of and anti-T of 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 chronic ly generated IFN-γ–producing CD8+ lymphocytes to the Tax protein, as reported recently by others (20).

This study establishes that CTLs against R of and T of 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 R of and T of peptide. Therefore, further experiments are needed to determine whether the cytotoxic response to R of and T of 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 R of and T of 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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