CD8⁺ T cells are an in vivo reservoir for human T-cell lymphotropic virus type I

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It is thought that human T-cell lymphotropic virus type I (HTLV-I), the first human retrovirus to be discovered, was isolated originally from the cultured CD4⁺ T lymphocytes of a patient with cutaneous T-cell lymphoma. Soon after, HTLV-III/lymphadenopathy-associated virus was identified by Tsuchida et al. in 1981. Since then, HTLV-I has been thought to preferentially infect CD4⁺ T cells in vivo. However, observations of HTLV-I proviral load in patients with HTLV-I–associated myelopathy/tropical spastic paraparesis suggest that HTLV-I may infect other cell types in addition to CD4⁺ T cells. To identify in vivo T-cell tropisms of HTLV-I, real-time quantitative polymerase chain reaction (PCR) and intracellular protein staining were used. A high amount of HTLV proviral DNA was detected from purified CD8⁺ T cells by quantitative PCR (between 1.64 and 62.83 copies of HTLV-I provirus per 100 isolated CD8⁺ T cells). CD8⁺ T cells expressed HTLV-I–related antigens (HTLV-I Tax and p19 protein) after a short time in cultivation. These results demonstrate that CD8⁺ T cells are also infected with HTLV-I and express HTLV-I antigens at levels that are comparable to HTLV-I–infected CD4⁺ cells. Therefore, CD8⁺ cells are an additional viral reservoir in vivo for HTLV-I and may contribute to the pathogenesis of HTLV-I–mediated disorders. (Blood. 2001;98:1858-1861) © 2001 by The American Society of Hematology

Introduction

Human T-cell lymphotropic virus type I (HTLV-I), the first human retrovirus to be discovered, was isolated originally from the cultured CD4⁺ T lymphocytes of a patient with cutaneous T-cell lymphoma. Soon after, HTLV-III/lymphadenopathy-associated virus was identified by Tsuchida et al. in 1981. Since then, HTLV-I has been thought to preferentially infect CD4⁺ T cells in vivo. However, observations of HTLV-I proviral load in patients with HTLV-I–associated myelopathy/tropical spastic paraparesis suggest that HTLV-I may infect other cell types in addition to CD4⁺ T cells. To identify in vivo T-cell tropisms of HTLV-I, real-time quantitative polymerase chain reaction (PCR) and intracellular protein staining were used. A high amount of HTLV proviral DNA was detected from purified CD8⁺ T cells by quantitative PCR (between 1.64 and 62.83 copies of HTLV-I provirus per 100 isolated CD8⁺ T cells). CD8⁺ T cells expressed HTLV-I–related antigens (HTLV-I Tax and p19 protein) after a short time in cultivation. These results demonstrate that CD8⁺ T cells are also infected with HTLV-I and express HTLV-I antigens at levels that are comparable to HTLV-I–infected CD4⁺ cells. Therefore, CD8⁺ cells are an additional viral reservoir in vivo for HTLV-I and may contribute to the pathogenesis of HTLV-I–mediated disorders. (Blood. 2001;98:1858-1861) © 2001 by The American Society of Hematology

Patients and methods

Patients

Five patients with HAM/TSP (HAM-1 to HAM-5) were tested. The diagnosis of HAM/TSP was made according to neurologic assessment and serologic testing for anti-HTLV-I antibody. All patients had diagnostic cerebral and spinal magnetic resonance imaging. All patients had anti-HTLV-I antibodies in serum and cerebrospinal fluid and had a slowly progressive spastic paraparesis. Informed consent was obtained from all patients.

Cell preparation

PBMCs were isolated from peripheral blood samples on a density gradient with lymphocyte separation medium (ICN Biomedicals, Aurora, OH), and

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the cells were viably cryopreserved in liquid nitrogen until tested. CD4+ T cells or CD8+ T cells were negatively selected from PBMCs with magnetic beads (MACS CD4+ or CD8+ T-cell isolation kit; Miltenyi Biotec, Auburn, CA) according to the manufacturer’s instructions.

**HTLV-I Tax and p19 expression in PBMCs**

PBMCs at 5 × 10^5 were placed in a culture well (round-bottom 96-well plate) in 200 μL RPMI-1640 supplemented with L-glutamine, penicillin, streptomycin, and 5% human AB serum. Harvested cells were washed with phosphate-buffered saline containing 1% fetal calf serum and 0.1% NaN₃ and incubated with anti-human CD4-phycoerythrin (Caltag Laboratories, Burlingame, CA) and anti-human CD8-TRICOLOR monoclonal antibodies (mAbs) (Caltag Laboratories) for 20 minutes at 4°C. Cells were then fixed and permeabilized with 4% formaldehyde and 0.1% saponin (CytoFix/Cytoperm Kits; Pharmingen, San Diego, CA) for 20 minutes at 4°C. After washing with 0.1% saponin buffer (Perm/Wash solution; Pharmingen), the cells were incubated with anti-HTLV-I Tax mAb (the reagent was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health; HTLV-I Tax transfection [168A51-42] from Dr Beatrice Langton) or anti–HTLV-I p19 mAb (Chemicon International, Temecula, CA) for 20 minutes at 4°C. After washing, fluorescein isothiocyanate–conjugated goat F(ab')₂-anti-mouse IgG2a or IgG1 mAb (Southern Biotechnology Associates, Birmingham, AL) was used as second antibody for labeling anti-HTLV-I Tax mAb or anti-HTLV-I p19 mAb, respectively. Flow cytometric analyses were performed using a FACS Calibur (Becton Dickinson, Mountain View, CA).

**Quantitative PCR**

HTLV-I proviral load was measured using an ABI PRISM 7700 Sequence Detector (Applied Biosystems) as described previously. DNA was extracted from 1 × 10^6 cells with the Puregene DNA Isolation Kit (Gentra, Minneapolis, MN) and was adjusted to 10 ng/μL. PCR conditions were as follows: 10 μL DNA solution was added to 40 μL reaction mixture containing 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 10 mM EDTA; 60 nM ROX (passive reference dye to 9 TA TCCGAA-3') positioned at 7276-7302 and 5'-CTCAGTGAGGATCTTCA TGAGGTAGT-3' positioned at 2146-2165 and 5'-CTCAGTGAGGATCTTCA TGAGGTAGT-3' positioned at 2250-2225. The Taqman fluorescent probe for HTLV-I pX region was 5'-TCCCAAGGTGGT-3' (performed under dye to normalize receptor signal); 5.5 mM MgCl₂; 0.2 mM each of dATP, dGTP, and dCTP; 400 μM dUTP; 0.5 U uracil-N-glycosylase; and 1.25 U Taq polymerase (AmpliTaq Gold; Applied Biosystems).

The primer set for HTLV-I pX region was 5'-AACAAAGTAAACACGCT-TATTATCAGC-3' positioned at 7276-7302 and 5'-ACACTGAGACTGG-TATCCGAA-3' positioned at 7355-7334. The primer set for β-actin was 5'-CACACAGTTGCACTACAGA-3' positioned at 2146-2165 and 5'-CTCAGTGAGGATCTTCA TGAGGTAGT-3' positioned at 2250-2225. The Taqman fluorescent probe for HTLV-I pX region was 5'-TCCCAAGGTGGT-3' performed under dye to normalize receptor signal; 5.5 mM MgCl₂; 0.2 μM each of primer; 0.1 μM Taqman probe; 200 μM each of dATP, dGTP, and dCTP; 400 μM dUTP; 0.5 U uracil-N-glycosylase; and 1.25 U Taq polymerase (AmpliTaq Gold; Applied Biosystems).

**Results**

**HTLV-I proviral load in purified T-cell subsets**

To determine the T-cell tropism of HTLV-I in vivo, we isolated CD4+ and CD8+ T cells from PBMCs of 5 HAM/TSP patients (purity of each T-cell subset is shown in Table 1) and quantified the amount of HTLV-I proviral DNA in each cell fraction by Taqman PCR. As shown in Figure 1, PBMCs from all HAM/TSP patients contained HTLV-I tax sequences in both CD8+ and CD4+ T cells. Between 1.64 and 62.83 copies of HTLV-I provirus per 100 isolated CD8+ T cells were observed (Figure 1). HTLV-I proviral DNA could not be amplified from HTLV-I–seronegative donors. This result strongly indicates that the HTLV-I–positive PCR signals of CD8+ T-cell fractions resulted from natural HTLV-I infection of CD8+ T cells in vivo. Given the high degree of purity (Table 1) and the extent of CD8+ infection, contaminating HTLV-I–infected CD4+ T cells in the purified CD8+ population (even assuming every CD4+ T cell was infected) could not account for the high HTLV-I proviral load observed in HAM/TSP CD8+ cells. Even CD8+ T cells from HAM/TSP patient no. 4, with the lowest proviral load (1.72 copies per 100 PBMCs), contained 1.64 copies of HTLV-I per 100 CD8+ T cells.

**HTLV-I Tax and p19 expression in PBMC**

To address the question of whether HTLV-I–infected CD8+ cells express HTLV-I antigen, we cultured PBMCs from HAM/TSP patients for a short time and measured the expression of HTLV-I Tax and p19 (HTLV-I Gag) proteins using an intracellular protein-staining technique. As reported previously, detection of HTLV-I antigen-expressing cells in uncultured HAM/TSP PBMCs was negligible (Figure 2, time 0) and was consistent with HTLV-I RNA analysis of fresh PBMCs from HAM/TSP patients. However, after briefly cultivating these PBMCs in vitro, we could detect HTLV-I Tax and p19 protein in both CD4+ and CD8+ T cells (Figures 2, 3). Maximum viral antigen expression occurred after 12 hours of cultivation and then declined (Figure 2). The HTLV-I Tax expression time course of CD4+ populations was similar to that of CD8+ cells, and all patients exhibited the same trend in protein expression. As expected from the HTLV-I proviral load data, the amount of HTLV-I proviral DNA in each cell fraction was quantified using the ABI PRISM 7700 Sequence Detector (Applied Biosystems) as described previously. DNA was extracted from 1 × 10^6 cells with the Puregene DNA Isolation Kit (Genta, Minneapolis, MN) and was adjusted to 10 ng/μL. PCR conditions were as follows: 10 μL DNA solution was added to 40 μL reaction mixture containing 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 10 mM EDTA; 60 nM ROX (passive reference dye to normalize receptor signal); 5.5 mM MgCl₂; 0.2 μM each of primer; 0.1 μM Taqman probe; 200 μM each of dATP, dGTP, and dCTP; 400 μM dUTP; 0.5 U uracil-N-glycosylase; and 1.25 U Taq polymerase (AmpliTaq Gold; Applied Biosystems).

| Table 1. Purity of isolated CD4+ or CD8+ T-cell fractions |
|-------------------------------|----------------|----------------|----------------|----------------|----------------|
| HAM-1                         | 99.13          | 97.21          | 98.07          | 98.02          | 96.27          |
| HAM-2                         | 0.07           | 0.01           | 0.23           | 0.03           | 0.1            |
| HAM-3                         | 95.58          | 94.95          | 92.64          | 94.53          | 97.5           |
| HAM-4                         | 0.16           | 0.08           | 0.66           | 0.26           | 0.2            |
| HAM-5                         |                |                |                |                |                |

The HTLV-I proviral load data in each cell fraction was measured by Taqman PCR. As shown in Figure 1, PBMCs from all HAM/TSP patients contained HTLV-I tax sequences in both CD8+ and CD4+ T cells. Between 1.64 and 62.83 copies of HTLV-I provirus per 100 isolated CD8+ T cells were observed (Figure 1). HTLV-I proviral DNA could not be amplified from HTLV-I–seronegative donors. This result strongly indicates that the HTLV-I–positive PCR signals of CD8+ T-cell fractions resulted from natural HTLV-I infection of CD8+ T cells in vivo. Given the high degree of purity (Table 1) and the extent of CD8+ infection, contaminating HTLV-I–infected CD4+ T cells in the purified CD8+ population (even assuming every CD4+ T cell was infected) could not account for the high HTLV-I proviral load observed in HAM/TSP CD8+ cells. Even CD8+ T cells from HAM/TSP patient no. 4, with the lowest proviral load (1.72 copies per 100 PBMCs), contained 1.64 copies of HTLV-I per 100 CD8+ T cells.

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show Tax expression in CD4

p19 expression of the cultured cells was measured at each time point. Graphs show Tax expression in CD4+ or CD8- T cells over time. The open circle (dotted line) indicates percentage of Tax expressing CD4+ T cells in total CD4+ T cells. The open square (solid line) indicates percentage of Tax expressing CD8- T cells in total CD8- T cells.

Figure 2. HTLV-I Tax expression in CD4+ or CD8- T cells. PBMC from four HAM/TSP patients were cultured for 0 hr, 12 hr, 24 hr, and 48 hrs. HTLV-I Tax and p19 expression of the cultured cells was measured at each time point. Graphs show Tax expression in CD4+ or CD8- T cells over time. The open circle (dotted line) indicates percentage of Tax expressing CD4+ T cells in total CD4+ T cells. The open square (solid line) indicates percentage of Tax expressing CD8- T cells in total CD8- T cells.

(Figure 1), the degree of Tax expression in CD8+ T cells was lower than in CD4+ T cells (Figure 2). No HTLV-I Tax expression was observed in cells from HTLV-I-seronegative donors.

To exclude the possibility that HTLV-I antigen expression in CD8+ cells (Figures 2, 3) was due to infection of these cells by HTLV-I-infected CD4+ cells during the short-term (12-48 hours) in vitro culture, we isolated purified CD8+ cells by negative selection from HAM/TSP PBMCs (more than 95% CD8+) and cultured them in vitro. After as little as 6 hours of in vitro incubation, HTLV-I Tax and p19 could be detected in these purified CD8+ cells (data not shown).

Percentage of HTLV-I infected CD4+ and CD8+ T cells expressing Tax

As an estimate of HTLV-I viral activity (viral protein expression) within these different T-cell populations, the proportion of peak Tax expression per infected cell was calculated for isolated CD4+ and CD8+ cells (Figure 4). Surprisingly, this analysis suggested that in 3 of 4 HAM/TSP patients, the amounts of HTLV-I expressed per HTLV-I-infected CD4+ or CD8+ T cell were similar, and in one

Ham/TSP patient (patient no. 4), the amount of HTLV-I expressed was even higher in CD8+ cells than in CD4+ cells (Figure 4).

Discussion

Although HTLV-I has been thought to preferentially infect CD4+ T cells in vivo,18 in vitro CD8+ cells could also be infected and immortalized.30,31 In this study, we demonstrate that in PBMC of HAM/TSP patients, CD8+ T cells have a high amount of HTLV-I proviral DNA (Figure 1). CD8+ T cells also express HTLV-I related antigens (HTLV-I Tax and p19 protein) after a short time in cultivation (Figures 2, 3). These results strongly indicate that HTLV-I infects CD8+ T cells in vivo and these naturally infected CD8+ T cells have the ability to produce HTLV-I antigens. Similar observation that short-cultured CD8+ T cells from HTLV-I-infected individuals (HAM/TSP patients and asymptomatic HTLV-I carriers) expressed HTLV-I Tax protein has also been recently reported.32 Interestingly, it has been shown that both CD4+ and CD8+ T cells subsets were equally susceptible to HTLV-2 infection.

High HTLV-I proviral loads have been demonstrated in patients with HAM/TSP32 and have been correlated with high immune responses, such as HTLV-I-specific cytotoxic T lymphocytes (CTLs)32 and anti-HTLV-I antibody.27 This elevated HTLV-I proviral load and high immune response have been suggested to play a role in HTLV-I–associated disease pathogenesis.35,36 It was originally believed that these high HTLV-I-specific immune responses were solely driven by HTLV-I–infected CD4+ T cells, which are elevated in patients with HAM/TSP.37 In vivo, HTLV-I–infected CD4+ T cells (helper T cells) were thought to become activated and present immunodominant viral peptides that stimulate virus-specific CD8+ CTLs. This high frequency of circulating HTLV-I–specific CTLs could then lyse these expanded, activated HTLV-I–infected CD4+ T cells, keeping the circulating viral infection in check.28 If virus-specific CTL recognition of antigen were to occur in a target organ containing HTLV-I–infected cells (heretofore thought only to be inflammatory HTLV-I–infected CD4+ T cells), then an immunopathologic process could ensue.35,36,38

The results in this report provide evidence that, similar to HTLV-I–infected CD4+ T cells, CD8+ T cells in PBMCs of HAM/TSP patients are infected with HTLV-I and are capable of
expressing viral protein, including HTLV-I Tax. This protein is known to transactivate both viral and host genes, including IL-2, IL-2 receptor (IL2r),22 and IL-15.39 The transactivation of IL-15 by HTLV-I Tax is of particular interest because this newly described cytokine has been reported to be involved in the maintenance and expansion of memory CD8+ T cells.40 HTLV-I–infected CD8+ T cells may therefore have a role as a significant viral reservoir in vivo and may also drive the high HTLV-I–specific immune response observed in patients with HAM/TSP. It remains to be seen whether comparable levels of HTLV-I–infected CD8+ cells are also observed in ATL patients. This newly reported CD8+ T-cell tropism should be studied to clarify the pathogenesis of HTLV-I–associated disease and has implications for the therapy of HTLV-I–mediated disorders and other human retroviruses.

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References

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