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Short Communication

Selected cytotoxic T lymphocytes with high specificity for HTLV-I in cerebrospinal fluid from a HAM/TSP patient

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Human T lymphotropic virus type I (HTLV-I)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a chronic inflammatory disease of the spinal cord in which HTLV-I Tax-specific cytotoxic T lymphocytes (CTL) have been suggested to be immunopathogenic. However, it is unknown whether the HTLV-I-specific CTL in the central nervous system differ from those in the periphery. We investigated functional T-cell receptor diversity in HTLV-I Tax11-19-specific CTL clones derived from peripheral blood and cerebrospinal fluid (CSF) of a HAM/TSP patient using analogue peptides of the viral antigen. CTL responses to the analogue peptides varied between T-cell clones, however, CTL clones from CSF showed limited recognition of the peptides when compared to those from peripheral blood. This suggests that CTL with highly focused specificity for HTLV-I Tax accumulate in the CSF and may contribute to the pathogenesis of HAM/TSP. Furthermore, this study provides a rationale for analogue peptide-based immunotherapeutic strategies focusing on the immunopathogenic T-cells in HTLV-I-associated neurologic disease. *Journal of NeuroVirology* (2002) **8,** 53–57.

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Introduction

Human T lymphotropic virus type I (HTLV-I) is the causative agent of HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Gessain *et al*, 1985; Osame *et al*, 1986, 1987). HAM/TSP patients show spastic gait and urinary disturbance with mild sensory disturbance (Osame *et al*, 1987). The disease is pathologically characterized by perivascular inflammatory cell infiltration with a predominance of CD8+ lymphocytes in the spinal cord (Umehara *et al*, 1993). In such lesions, HTLV-I-specific CD8+ cytotoxic T lymphocytes (CTL) and expression of HTLV-I gene products have also been shown (Moritoyo *et al*, 1996; Levin *et al*, 1997). HTLV-I Tax11-19 is an immunodominant epitope in human leukocyte antigen (HLA)-A*0201 HAM/TSP patients, in which the frequency of circulating HTLV-I Tax11-19-specific CD8+ T-cells can be as high as 14% of the CD8+ T-cell population (Jacobson *et al*, 1990; Kubota *et al*, 1998). Importantly, the frequency of virus-specific T-cells is even higher in the cerebrospinal fluid (CSF) than in the peripheral blood of HAM/TSP patients, suggesting that HTLV-I-specific CTL may accumulate in inflammatory lesions (Greten *et al*, 1998). These studies support the view that the virus-specific CD8+ CTL may have an immunopathogenic role in the inflammatory lesions of HAM/TSP patients (Jacobson, 1996).

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To design an immunotherapeutic strategy focusing on T-cell receptor (TCR) of the pathogenic T-cells in HAM/TSP, it is crucial to know if there is skewed TCR usage in the inflammatory site. Sequencing analysis has revealed heterogeneity of TCR in HTLV-1 Tax-specific CTL clones from peripheral blood mononuclear cells (PBMC) (Utz *et al*, 1996). Also, in patients with multiple sclerosis (MS), it has been shown that the TCR sequence repertoire in myelin basic protein-specific T-cells is diversified (Utz *et al*, 1994). However, recent immunological studies have clearly shown that T-cells with structurally different TCR can recognize the same antigen (Hemmer *et al*, 1997; Ding *et al*, 1998; Gran *et al*, 1999). Therefore, it is clinically relevant to define functional TCR diversity rather than structural TCR diversity of potentially immunopathogenic T-cells. Altered peptide ligands (APL) are analogue peptides modified at TCR contact residues of an antigenic peptide, which can act as a TCR agonist to a partial agonist or an antagonist for given T-cells (Evavold and Allen, 1991; Kubota *et al*, 2000). Therefore, APL may have the potential to manipulate T-cell function *in vivo* and to alter the outcome in human diseases such as MS, as recently demonstrated (Bielekova *et al*, 2000; Kappos *et al*, 2000). Furthermore, APL can be used as a powerful tool to study detailed functional T-cell specificity to the antigen (Hemmer *et al*, 1997; Kubota *et al*, 2000). In this study, we compared functional TCR diversity in HTLV-I Tax-specific CTL clones derived from PBMC and CSF of a HAM/TSP patient using APLs of the viral antigen.

To compare HTLV-I Tax-specific TCR diversity between cells in the CSF and peripheral blood, a panel of T-cell clones were generated from an HLA-A*0201 HAM/TSP patient known to have strong HTLV-I Tax11-19-specific T-cell response in these compartments (Greten *et al*, 1998; Kubota *et al*, 1998). The HLA-A*0201 HAM/TSP patient had typical neurological symptoms with 19 years' duration of disease and HTLV-I seropositivity confirmed by Western blot as described previously (Osame *et al*, 1987; Greten *et al*, 1998). Lymphocytes were isolated from heparinized peripheral blood by Ficoll-Hypaque centrifugation. Cerebrospinal fluid was obtained atraumatically. CD8+ cells from the PBMC were seeded at a concentration of $0.3–1.0 \times 10^2$ cells per 100 wells in a 96-well U-bottom plate with irradiated PBMC prepulsed with HTLV-I Tax11-19. CSF cells were directly seeded into wells at a concentration of $0.3–1.0 \times 10^2$ cells per 100 wells in the same culture condition. Fourteen HTLV-I Tax11-19-specific CTL clones from peripheral blood (PBMC-CTL) and 13 CTL clones from CSF (CSF-CTL) were established.

The CTL assay was performed using Europium, a lanthanide whose chelates of bis(acetoxymethyl)-2,2′,6′,2″-terpyridine-6,6″-dicarboxylate (BATDA) have a long fluorescence decay time used for time-resolved fluorometry, as previously described (Kubota *et al*, 2000). Briefly, HLA-A*0201-transfected target cells were labeled with Europium, and pulsed with 1 μM HTLV-I Tax11-19 or APLs. Three thousand target cells were transferred to wells in a 96-well plate. The same number of effector cells in medium, medium alone, or 1% Triton X-100 was added to the wells. After a 3-h incubation at 37°C, the fluorescence intensity of the chelates formed by Europium and the fluorescence-enhancing ligand was measured by a fluorometer (DELFIA1234; Wallac). The percent specific lysis was calculated with the following formula: (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100. Relative CTL response to APL against to HTLV-I Tax11-19 was given by the following formula: (specific lysis to APL)/(specific lysis to HTLV-I Tax11-19) × 100. The assay was conducted in triplicate. Analysis of variance (ANOVA) with Bonferroni correction was the statistical method used to compare the specific lysis for APL with that for native Tax11-19 peptide in PBMC or CSF-derived CTL clones. Mann–Whitney *U*-test was used to compare the relative CTL response in PBMC-CTL clones with that in CSF-CTL clones to each APL. *P* values less than 0.05 were considered statistically significant. Initially, titration of the native HTLV-I Tax11-19 peptide demonstrated that CTL responses of each T-cell clone reached a plateau at 10 nM (data not shown). Therefore, all CTL assays were performed using 1 μM of peptide. To test whether killing activities to HTLV-I Tax11-19-prepulsed targets differed between clones derived from PBMC and CSF, specific lysis of HTLV-I Tax peptide-pulsed targets were measured. The percent specific lysis to HTLV-I Tax11-19 was not statistically different between PBMC-CTL and CSF-CTL (mean ± SD were 51.3 ± 15.6% and 54.2 ± 12.8%, respectively, data not shown), suggesting that there was no apparent difference in cytolytic activity to the Tax peptide between CTL in the periphery and in the CSF.

The amino acid sequence of HTLV-I Tax11-19 is LLFGYPVYV (Kubota *et al*, 1998). We synthesized L-alanine-substituted peptides at positions 4, 5, 6, and 8, which are expected as TCR binding positions (Ding *et al*, 1998). They were designated as G4A, Y5A, P6A, and Y8A, respectively. Influenza virus M1 peptide (GILGFVFTL) was used as a control peptide that binds to HLA-A2 (Utz *et al*, 1996). To determine functional TCR diversity in CTL derived from PBMC and CSF, CTL responses to a panel of APL were determined for each T-cell clone. Figure 1 represents CTL responses to native Tax11-19 peptide and APL pulsed targets from 2 HTLV-I Tax11-19-specific CTL clones. PBMC-derived T-cell clone N1104 strongly recognized targets pulsed with native Tax peptide, G4A, and P6A; moderately recognized targets pulsed with Y8A; and weakly lysed targets pulsed with Y5A. By contrast, clone N722 derived from CSF only recognized the native Tax11-19 peptide-pulsed targets. As shown in Figure 2, analysis of a larger panel of PBMC- and CSF-CTL clones demonstrated that

CTL recognition of APL varied between T-cell clones. Results are plotted as cytolytic responses of CTL to APL pulsed targets relative to the native Tax11-19 response. In PBMC-CTL clones, only the recognition of Y5A and Y8A was significantly decreased as compared to that of the native Tax 11-19 peptide (asterisk in Figure 2), while in CSF-CTL clones, the recognition of all the APL were decreased (asterisk in Figure 2). Moreover, when we compared the relative CTL response to APL between PBMC- and CSF-CTL clones, the average of the CTL responses to all the APL in the CSF-CTL clones were lower than those from the PBMC-derived CTL clones, and the recognition of P6A and Y8A was significantly decreased in CSF-CTL clones (Figure 2). This indicates that CTL responses to APL in CSF-derived T-cell clones may not be tolerated well as compared to those derived from the PBMC, suggesting that CTL with restricted recognition for HTLV-I Tax11-19 accumulate in the CSF of the HLA-A*0201 HAM/TSP patient.

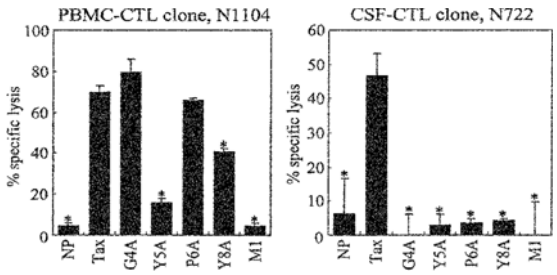

Figure 1 Representative specific lysis of HTLV-I Tax11-19- and APL-pulsed targets by HTLV- I Tax11-19-specific CTL clones N1104 and N722 established from peripheral blood and CSF, respectively (mean ± SD). G4A, Y5A, P6A and Y8A are analogue peptides of HTLV-I Tax11-19 in which a single amino acid is substituted by alanine (e.g., in G4A, glycine at fourth position of the HTLV-I Tax11-19 is exchanged to alanine). M1 is a control peptide derived from influenza virus that binds to HLA-A*0201 and NP indicates no peptide. Asterisk indicates that the specific lysis to APL is significantly decreased when compared to that to HTLV-I Tax11-19 in each T-cell clone. CTL assay was conducted in triplicate at the effector to target ratio of 1 to 1.

Possible explanations for why HTLV-I-specific T-cells with increased specificity accumulate in the CSF include: 1) antigen-specific T-cells with highly focused specificity could be recruited into the central nervous system (CNS) from the periphery or 2) T-cells with enhanced specificity to the antigen could readily expand in the CNS. Further studies including comparisons of heterogeneity in the viral sequences and of viral activation state between CTL in

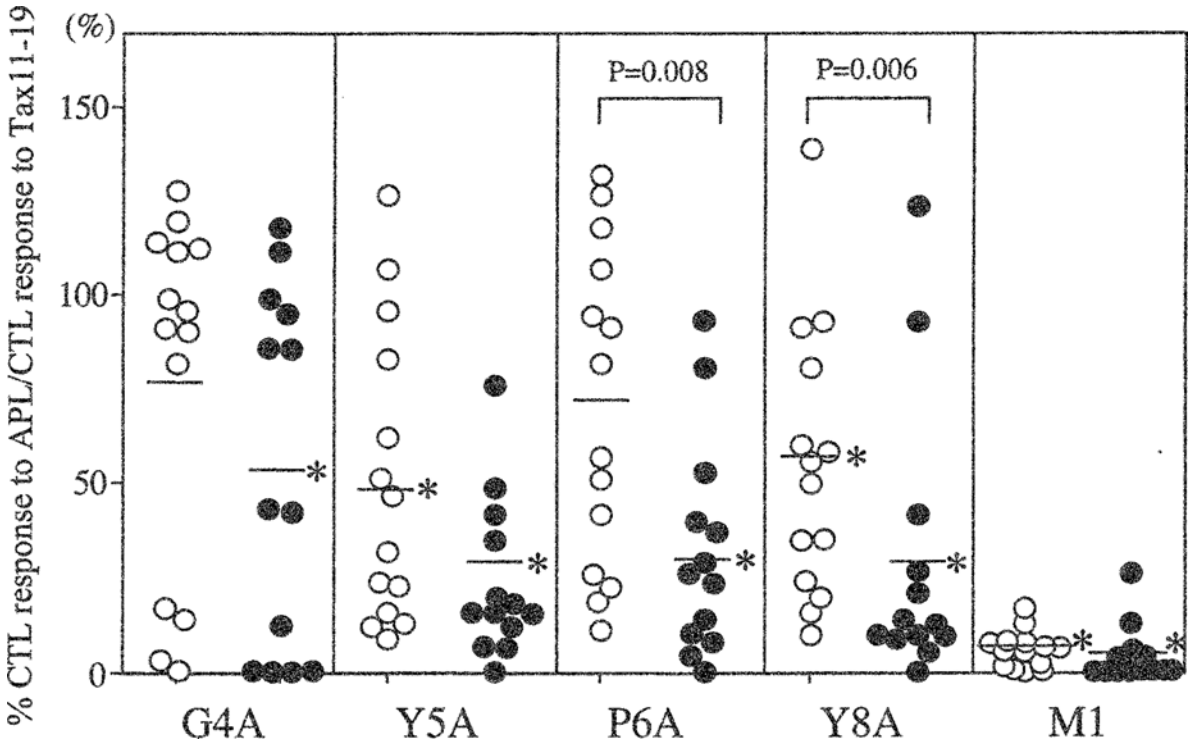

Figure 2 CTL responses to APLs in HTLV-I Tax11-19-specific CTL clones derived from peripheral blood and CSF of a HAM/TSP patient. Open and closed circles indicate relative CTL responses of T-cell clones from peripheral blood ($n=14$) and CSF ($n=13$), respectively. The relative CTL response is defined as the lysis of a given CTL clone to an APL-pulsed targets divided by the lysis of the clone against the HTLV-I Tax11-19-pulsed targets. Asterisk indicates that the percent specific lysis to APL is significantly decreased when compared to that to HTLV-I Tax11-19 in PBMC- or CSF-CTL clone groups (by ANOVA with Bonferroni correction, $P < 0.0033$). The horizontal bar indicates the average of relative cytolysis to APL in each clone group. The recognition of P6A and Y8A was significantly decreased in CSF-CTL clones compared with that in PBMC-CTL clones by Mann–Whitney U-test ($P = 0.008$ and $P = 0.006$, respectively). The percent specific lysis to HTLV-I Tax11-19 ranged from 23 to 81% and was not different between PBMC- and CSF-CTL clones (mean ± SD were 51.3 ± 15.6% and 54.2 ± 12.8%, respectively).

PBMC and CNS would be needed to distinguish between these possibilities. It has been reported that frequency of HTLV-I-specific CD8+ T-cells is higher in CSF than in the periphery and that HTLV-I-infected CD4+ cells expressing HTLV-I Tax protein are increased in CSF when compared to that in PBMC of HAM/TSP patients (Greten *et al*, 1998; Moritoyo *et al*, 1999). Therefore, both HTLV-I-infected CD4+ cells and virus-specific CD8+ CTL accumulate in the CNS of HAM/TSP patients and are activated at the inflammatory site. In such lesions, CTL with highly focused specificity for HTLV-I may play a crucial role in the pathogenesis of the disease. If so, inactivation or depletion of the HTLV-I-specific CTL in the inflammatory site may be clinically beneficial.

A large body of evidence indicates that T-cell recognition is degenerate and more promiscuous than previously considered (Evavold and Hemmer, 1991; Hemmer *et al*, 1997; Gran *et al*, 1999). This opens the possibilities that modifications of T-cell functions by APL may result in increased activation or inhibition of these functions and potentially alter the outcome of immune-mediated human diseases. However, heterogeneity of TCR repertoire of antigen-specific T-cells in immune-mediated diseases such as MS may make it difficult to design an immunotherapy targeting TCR/antigen/HLA complex (Utz *et al*, 1994; Bielekova *et al*, 2000; Kappos *et al*, 2000). In HAM/TSP patients, the TCR sequence analysis of the peripheral HTLV-I Tax11-19-specific T-cells revealed that TCR usage is diversified (Utz *et al*, 1996). In our patient, HTLV-I Tax-specific CTL demonstrated diverse recognition of APL in PBMC. However, CTL from the CSF had enhanced fine specificity for the antigen as compared to those from the periphery. Therefore, a TCR-targeting therapy may be appropriate in HTLV-I-associated neurologic disease.

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