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Target Epitope in the Tax Protein of Human T-Cell Leukemia Virus Type I Recognized by Class I Major Histocompatibility Complex-Restricted Cytotoxic T Cells

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A *trans*-acting regulatory gene product $p40^{tax}$ (Tax) of human T-cell leukemia virus type I (HTLV-I) is one of the main target antigens recognized by cytotoxic T lymphocytes (CTL) specific for HTLV-I. A CTL epitope within the Tax protein was identified in this report. HTLV-I-specific $CD8^+$ CTL lines established from two HTLV-I carriers with HTLV-I-associated myelopathy or Sjögren syndrome were previously demonstrated to kill predominantly the target cells expressing HTLV-I Tax. The CTL from two patients showed significant levels of cytotoxicity to autologous target cells pulsed with a synthetic peptide of 24 amino acids corresponding to the amino-terminal sequences of the Tax protein. Allogeneic target cells were also sensitized for CTL by this peptide when the target cells have HLA-A2. Tax-specific cytotoxicity, detected as cytolysis of the target cells infected with vaccinia virus-HTLV-I recombinant expressing Tax protein, was almost completely inhibited by competitor cells pulsed with the synthetic peptide. This indicates that a major CTL epitope is present in this peptide. Further analysis using shorter peptides revealed that the core sequence of the CTL epitope was LLFGYPVYV at positions 11 through 19. This sequence can be aligned with the HLA-A2-specific motifs reported recently.

Human T-cell leukemia virus type I (HTLV-I), the causative agent of adult T-cell leukemia (11, 22), is also closely related to HTLV-I-associated myelopathy or tropical spastic paraparesis (HAM/TSP) (6, 21). A variety of autoimmune-like disorders including T-cell alveolitis, myopathy, uveitis, arthritis, and Sjögren syndrome are seen in HTLV-I-infected individuals as well (9, 20, 26, 28), although the precise relationship between these disorders and HTLV-I infection is undetermined. HAM/TSP is a chronic myelopathy associated with elevated levels of HTLV-I-specific antibodies in the peripheral blood and cerebrospinal fluid. The level of cellular immunity against HTLV-I is also elevated in these patients, detected as in vitro spontaneous proliferation (13) and HTLV-I-specific cytotoxicity (15) of peripheral blood lymphocytes (PBL). Despite this pronounced immune response, HTLV-I proviral DNA and RNA can be detected in the PBL of HAM/TSP patients more frequently than in those of asymptomatic HTLV-I carriers (7, 8, 31). The elevated levels of anti-HTLV-I immunity in HAM/TSP are presumed to be the result of active stimulation with HTLV-I in these patients.

A T-cell immune response damaging host cells has been well characterized in animal models such as virus-induced lymphocytic choriomeningitis (LCM) in mice (3). Evidence that T-cell-deficient mice do not develop disease as a result of LCM virus infection and that LCM can be induced in these animals by adoptively transferring cloned class I major histocompatibility complex (MHC)-restricted cytotoxic T lymphocytes (CTL) specific for LCM virus, indicates that these CTL are immunopathological effectors in LCM (2). Although whether HTLV-I-specific CTL play a similar role in HAM/TSP is still controversial, the presence of lymphocyte infiltration in the spinal cord lesions of HAM/TSP patients (1) suggests that cellular immune mechanisms may be involved in the pathogenesis of this condition.

HTLV-I-specific CTL can be induced from PBL of human HTLV-I carriers by in vitro stimulation with HTLV-I antigen (18, 19). We previously demonstrated, by using vaccinia virus-HTLV-I recombinants, that $CD8^+$ CTL from HTLV-I carriers with HAM/TSP or Sjögren syndrome showed cytotoxicity to the target cells expressing various HTLV-I proteins, especially Tax, in a class I MHC-restricted manner (17). Other investigators have reported that cytotoxicity to the target expressing Tax is readily detectable in the PBL of HAM/TSP patients (15). The HTLV-I Tax (or Tax-induced protein) is, therefore, likely to be one of the major target antigens for the CTL response in these HTLV-I-infected individuals.

In the present study, fine mapping of the CTL epitope within the HTLV-I Tax protein recognized by CTL induced from HTLV-I carriers with autoimmunelike disorders and the association of this epitope with class I MHC molecules are described.

MATERIALS AND METHODS

Induction of HTLV-I-specific CTL. HTLV-I-specific CTL lines were established from the PBL of HTLV-I carriers, as described previously (19). One of the two donors has symp-

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toms characteristic of HAM/TSP (patient 1), and the other has Sjögren syndrome (patient 2). The PBL of these individuals were repeatedly stimulated in vitro with autologous HTLV-I-infected T cells treated with mitomycin and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 10 ng of recombinant interleukin-2 (Shionogi, Osaka, Japan) per ml. These cell lines were $CD8^+$ and showed HLA-A2-restricted cytotoxicity specific for HTLV-I, especially for HTLV-I Tax protein (17). These CTL lines were utilized as effectors for further experiments between 2 and 4 months after the initiation of the culture.

Cell lines. LCL#1 and LCL#2 are Epstein-Barr virus-transformed B-cell lines originating from the donors noted above. HAS15, BTB, AKIBA, TOK, TF6, and TS10 are Epstein-Barr virus-transformed human B-cell lines and were distributed in the 10th International Histocompatibility Workshop. These autologous and allogeneic B cells were used as targets of cytotoxicity assays. Autologous T-cell lines infected with HTLV-I were established by mass cultivation of phytohemagglutinin-stimulated PBL of patients 1 and 2 in the presence of interleukin-2.

Synthetic peptides. Peptides were synthesized as amino acids by the stepwise method of solid-phase peptide synthesis on an automated peptide synthesizer (model 430A; Applied Biosystems, Inc., Foster City, Calif.) with chemicals and program cycles provided by the manufacturer, deprotected and cleaved from the supporting resin with hydrogen fluoride, and purified to greater than 95% purity by reverse-phase chromatography on an HPLC system (model SPD-6AV; Shimazu Corporation, Kyoto, Japan). Amino acid analysis of all synthetic peptides was performed on an amino acid analyzer (model L-8500; Hitachi, Ltd., Tokyo, Japan) with ninhydrin derivatization and ion-exchange chromatography according to the manufacturer's specifications, and it confirmed that all the peptides used in this study had the expected amino acid content.

Recombinant vaccinia virus. The vaccinia virus-HTLV-I recombinant virus LO5-40x contains a DNA fragment with the *tax* gene of HTLV-I inserted into vaccinia virus (LO5 strain) at the hemagglutinin (HA) coding region by in vivo recombination (25). Target cells were infected with LO5-40x at 50 PFU per cell at 37°C for 18 h and radiolabeled for cytotoxicity assays. Infection with the recombinant vaccinia viruses was evaluated by the indirect immunofluorescence staining method using rabbit serum to whole vaccinia virus antigens. More than 70% of the target cells were infected with LO5-40x. Expression of HTLV-I $p40^{tax}$ antigens was confirmed by immunoblot and immunofluorescence methods.

Cytotoxicity assay. Cell-mediated cytotoxicity assays were performed by the ^{51}Cr release method (19). Radiolabeled target cells were aliquoted into a microplate at 10^4 cells per well. These cells were next preincubated with synthetic peptides at a final concentration of 10 μM at 37°C for 1 h, prior to incubation with various numbers of effector cells for 4 h. The radioactivity released in the supernatants was measured, and the percentage of specific cytotoxicity was calculated as follows: (release in test − spontaneous release)/(maximal release − spontaneous release) × 100. The average spontaneous release for the cytotoxicity assays was 29%.

RESULTS

CTL response to HTLV-I Tax. CTL lines were induced from patient 1 (HAM/TSP) and 2 (Sjögren syndrome) by repeated stimulation with HTLV-I-infected cells in vitro. The cytotoxic specificities of the CTL line and the freshly isolated PBL from patient 1 are shown in Fig. 1. The CTL line showed significant cytotoxicity against autologous HTLV-I-infected T cells and autologous B cells infected with recombinant vaccinia virus expressing HTLV-I Tax (Fig. 1A). These CTL also showed low levels of cytotoxicity to the target cells expressing HTLV-I envelope or core proteins, but not those infected with the control vaccinia virus. In contrast, PBL freshly isolated from the same donor showed minimal levels of cytotoxicity against autologous target cells, regardless of the expression of HTLV-I antigens (Fig. 1B). Similar results were obtained with a CTL line derived from patient 2 (Fig. 1C and D). The CTL from this patient primarily killed autologous B cells expressing Tax as well as HTLV-I-infected T cells. They also showed significant levels of cytotoxicity to the target cells expressing envelope proteins, but minimally lysed the targets expressing core proteins. Freshly isolated PBL from this subject did not show HTLV-I-specific cytotoxicity either. These observations indicated that a small number of CTL present in the PBL were activated and enriched in the culture by in vitro stimulation with HTLV-I antigens and that these CTL are capable of killing the target cells expressing HTLV-I antigens, especially Tax protein.

Mapping of HTLV-I Tax-specific CTL epitope. To identify target epitopes recognized by HTLV-I Tax-specific CTL, we examined the cytotoxic response of the CTL lines against autologous B cells in the presence of synthetic peptides with the amino acid sequence of HTLV-I Tax (23). Initially we prepared a series of 24-amino-acid synthetic peptides corresponding to the N-terminal half of the Tax protein. Among these peptides, CTL lines from both patients lysed target cells more effectively in the presence of peptide Tax1-24 than any other peptide tested (Fig. 2). The predominance of Tax1-24 in sensitizing targets was reproducible in repeated experiments. Tax1-24 alone did not show any significant cytotoxicity. Both CTL lines also showed low levels of cytotoxicity to the targets in the presence of peptide Tax177-200. However, the significance of this peptide in sensitizing targets was unclear, as Tax177-200 itself was slightly toxic to the target cells.

Inhibition of Tax-specific cytotoxicity by unlabeled target cells treated with synthetic peptides. We then assessed to what extent peptide Tax1-24 shared the target epitopes for Tax-specific CTL. Cytotoxicity against radiolabeled autologous B cells infected with recombinant vaccinia virus (LO5-40x) expressing Tax protein was examined in the presence of unlabeled B cells treated with several synthetic peptides including Tax1-24 (Fig. 3). More than 90% of the Tax-specific cytotoxicity was inhibited with the competitor B cells sensitized by Tax1-24 at competitor-to-target ratios of 20 and 40. Competitor cells treated with the other peptides tested (Tax33-56, Tax91-104, and Tax177-200) or untreated competitor cells showed little effect on the cytotoxicity. Unlabeled competitor cells infected with LO5-40x as positive controls completely inhibited Tax-specific cytotoxicity under the same conditions. These results indicate that peptide Tax1-24 possesses an epitope recognized by HTLV-I Tax-specific CTL, which shares most of the target epitopes on the Tax protein expressed by the recombinant vaccinia virus. Thus, the predominant CTL epitope exists in the N-terminal sequences of HTLV-I Tax.

MHC restriction of cytotoxicity against Tax peptides. We previously reported that the Tax-specific cytotoxicity of the CTL from patients 1 and 2 was restricted by HLA-A2. To

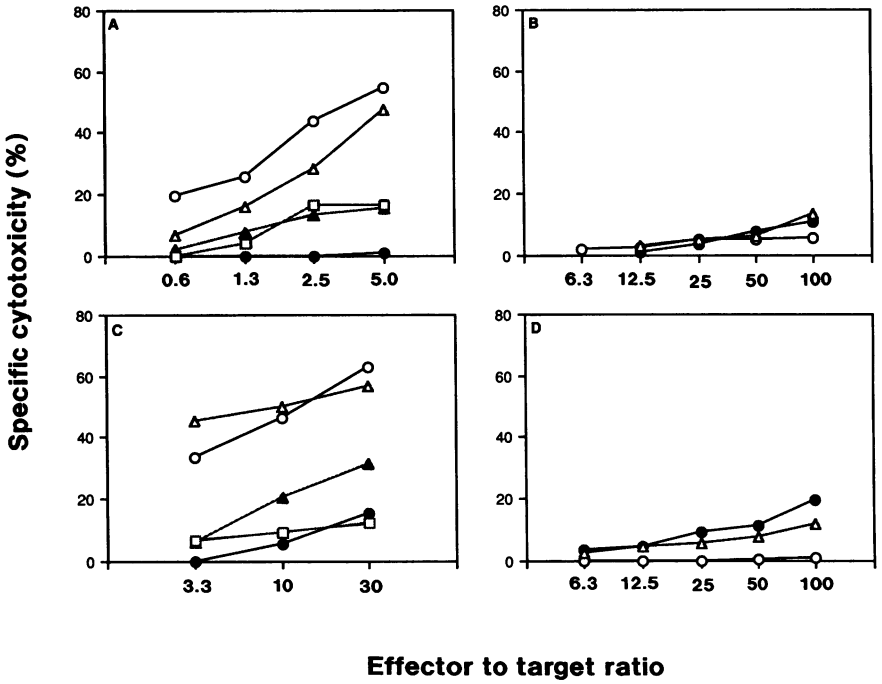

FIG. 1. Cytotoxic specificities of the CTL line from patients 1 (A) and 2 (C) and of freshly prepared PBL of patients 1 and 2 (B and D), respectively. The target cells used were autologous HTLV-I-infected T cells (○), autologous B cells infected with control vaccinia virus (●), and autologous B cells infected with vaccinia virus-HTLV-I recombinants expressing HTLV-I Tax (△), envelope (▲), or core (□) proteins. HTLV-I-infected T-cell lines contained about 90% positive cells for HTLV-I antigens. The vaccinia virus-HTLV-I recombinant virus infected 68% of the target B cells on average.

verify the class I MHC restriction of the cytotoxicity against peptide Tax1-24, various allogeneic B cells were utilized as targets in the presence of Tax1-24. Table 1 shows the reactivity of CTL from patient 1 against autologous or allogeneic target cells treated with either the vaccinia virus recombinant (LO5-40x), peptide Tax1-24, or control medium. The CTL killed LO5-40x-infected targets sharing HLA-A2 more effectively than the ones sharing HLA-A24, HLA-DR7, or HLA-DR12. This preferential cytotoxicity to the HLA-A2-positive targets was even more prominent among the targets treated with Tax1-24. Only the target B cells possessing HLA-A2 were killed by the CTL in the presence of Tax1-24. None of the target cells were susceptible to the CTL without Tax1-24 treatment of LO5-40x infection. Similar results were obtained when using CTL from patient 2, which also possessed HLA-A2. Thus, the cytotoxicity to the targets sensitized with Tax1-24 appeared to be restricted by HLA-A2. The clear correlation between the ability of the peptide to sensitize targets and the presence of HLA-A2 in the targets suggests that Tax1-24 may contain a sequence that binds to the HLA-A2 molecule.

Fine mapping of CTL epitope in the HTLV-I Tax protein. We then examined smaller synthetic peptides for the ability of sensitizing targets to identify a CTL epitope within peptide Tax1-24. Recently, detailed HLA-A2-specific motifs of antigenic peptides expressed in the grooves of class I MHC molecules have been revealed (4). These peptides are nonapeptides with two anchor residues, namely, Leu or Met at position 2 and Val or Leu at position 9. In the overall amino acid sequences of HTLV-I Tax, such motifs were

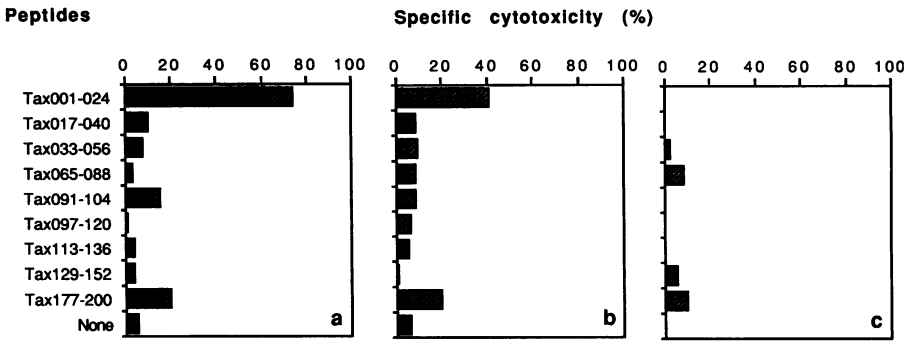

FIG. 2. Screening of HTLV-I synthetic Tax peptides for the ability to sensitize targets to CTL. Specific cytotoxicity of CTL from patients 1 (a) and 2 (b) was examined against autologous B cells in the presence of 10 μM of the synthetic peptides indicated. The effector-to-target ratio was 10. Cytolysis of the B cells alone in the presence of the synthetic peptides was also measured (c).

TABLE 1. Human leukocyte antigen restriction of HTLV-I Tax-specific cytotoxicity

CTL donor	Target cells[a]	Matched HLA antigens[b]	Specific cytotoxicity[c] (%) to the target cells treated with:			
			LO5-40x	LO5-HA$^-$	Tax1-24	Medium
Patient 1	LCL#1	Autologous	30	1	32	5
	HAS15	A2, B54, C1, C3	33	5	44	−1
	BTB	A2, C1	38	10	62	2
	AKIBA	A24	19	4	4	−4
	TOK	A24	6	NT	4	−3
	TF6	DR7, DR12	3	0	1	−1
	TS10	None	NT	NT	5	1
Patient 2	LCL#2	Autologous	40	2	56	4
	HAS15	A2, B5, C1	50	10	27	3
	BTB	A2, C1	46	7	57	5
	TS10	A26, DR4	0	1	3	2
	AKIBA	DR2	0	4	1	−2
	TF6	None	2	0	−1	−1

[a] All the targets used are Epstein-Barr virus-transformed B cells.
[b] Common HLA antigens in target cells and the CTL were listed.
[c] Cytotoxicity of CTL from patient 1 and 2 was examined against each target after treatment with recombinant vaccina viruses (LO5-40x and LO5-HA$^-$), synthetic peptide (Tax1-24), or medium (control). The effector-to-target ratio was 10. NT, not tested.

found in the residues 11 to 19 and 178 to 186. Residues 11 to 19 are present in Tax1-24, the peptide able to sensitize targets, and residues 178 to 186 are present in Tax177-200, which showed slight cytotoxicity as indicated in Fig. 2. We prepared a series of 9-amino-acid synthetic peptides, including residues 11 to 19 and 178 to 186, and examined their ability to sensitize targets. Amino acid sequences of the synthetic peptides prepared and the results of the cytotoxicity assays using these peptides are summarized in Table 2. The target cells incubated with peptides Tax10-18 and Tax11-19 were the most susceptible to the CTL from both patients 1 and 2. Tax8-16 and Tax8-17, showed little effect. Tax12-20 and Tax13-21 also sensitized targets, especially for the CTL of patient 1. In contrast, the target cells treated with peptide Tax177-185, Tax177-186, or Tax179-187 were not lysed by the CTL.

To assess the affinity of the peptides to CTL, a titration experiment was performed (Fig. 4). This revealed that 1 nM of peptide Tax11-19 was sufficient to sensitize targets. A concentration of 1 μM of Tax10-18 was required to obtain a similar effect. CTL lysed target cells in the presence of Tax12-20 or Tax13-21 only at the highest concentration tested (10 μM). Tax11-19, the peptide with the strongest affinity to CTL, did not show any cytotoxicity when the peptide alone was added to the target cells. Thus, the amino acid sequence LLFGYPVYV appears to be the core sequence of the CTL epitope.

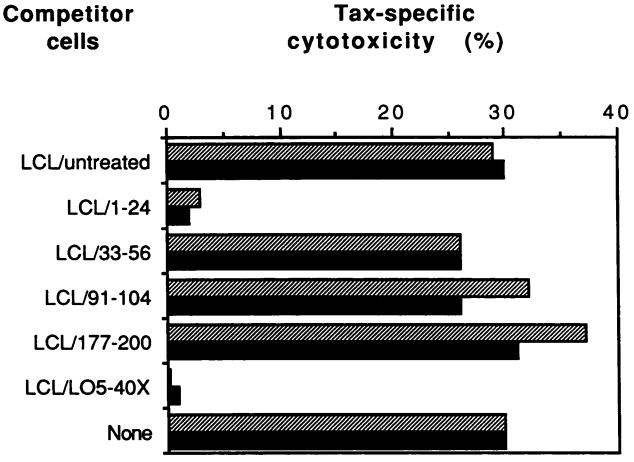

FIG. 3. Inhibition of cytotoxicity to the target cells expressing tax protein by competitor cells pulsed with synthetic peptides. Cytotoxicity of CTL from patient 1 against autologous B cells infected with recombinant vaccinia virus LO5-40x was measured in the presence of competitor cells. Unlabeled competitor cells were pretreated with either 10 μM of the Tax peptides indicated, LO5-40x, or control medium at 37°C for 18 h, washed, and then used as competitor cells. The effector-to-target ratio was 10. The competitor-to-target ratio was 20 (▨) or 40 (▩).

TABLE 2. Fine mapping of Tax CTL epitope and the HLA-A2-specific dominant anchor residues

Peptide	Sequence	Specific cytotoxicity[a] (%) to the sensitized targets by the CTL of:	
		Patient 1	Patient 2
Tax8-16	GQSLLFGYP	4	7
Tax8-17[b]	GQSLLFGYPV	3	10
Tax10-18	SLLFGYPVY	40	59
Tax11-19	LLFGYPVYV	42	65
Tax12-20	LFGYPVYVF	36	14
Tax13-21	FGYPVYVFG	39	23
Tax177-185	GQLGAFLTN	−2	0
Tax177-186[b]	GQLGAFLTNV	1	2
Tax179-187	LGAFLTNVP	−2	2
HLA-A2-specific dominant anchor residues[c]	*L******V		

[a] Specific cytotoxicity of CTL from patient 1 and 2 was examined against autologouso B cells in the presence of 10 μM of the various peptides indicated. The effector-to-target ratio was 10.
[b] Tax8-17 and Tax177-186 are 10-amino-acid peptides because of the difficulty of placing Q at the N terminus.
[c] According to reference 4. The asterisks indicate the positions which do not have any dominant residues.

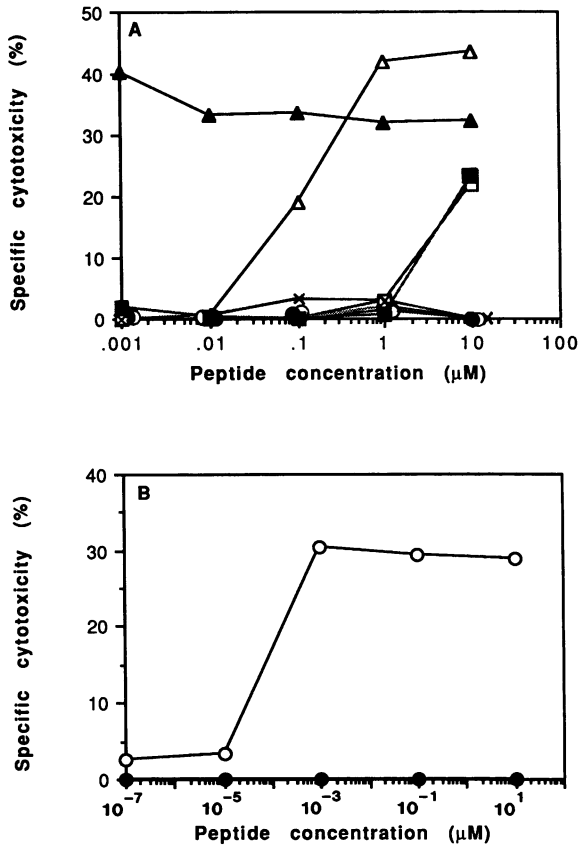

FIG. 4. (A) Titration of the peptides for the ability to sensitize targets. Cytotoxicity of the CTL from patient 1 to autologous B cells was examined in the presence of serially diluted peptides Tax8-16 (○), Tax8-17 (●), Tax10-18 (△), Tax11-19 (▲), Tax12-20 (□), Tax13-21 (■), and Tax177-186 (X). The effector-to-target ratio was 10. (B) Cytotoxic effects on the target cells at further dilutions of Tax11-19 with (○) or without CTL (●). The combination of CTL and targets utilized was the same as above. The effector-to-target ratio was 5.

DISCUSSION

$CD8^+$ CTL recognize target antigens associated with particular MHC class I molecules. Crystallography of these MHC molecules revealed a groove containing heterogenous peptide-sized material considered to represent antigen peptides recognized by T lymphocytes (5). Recently, important information about allele-specific motifs of T-cell epitope was provided by sequencing of self-peptides eluted from MHC molecules (4). It appears that HLA-A2-restricted epitopes are nonapeptides with Leu or Met at position 2 and Val or Leu at position 9, although not all of the HLA-A2-restricted epitopes previously reported are completely aligned to this motif. The CTL epitope within HTLV-I Tax identified in the present study corresponds to this motif. The amino acid sequences at positions 11 through 19 contain Leu at position 12 and Val at position 19, which can be aligned to the HLA-A2-restricted motif. The synthetic peptide Tax11-19 sensitized target cells with prominent affinity to CTL. The affinity of Tax10-18, which lacks Val at position 19, was lower than that of Tax11-19 but higher than that of Tax12-20 or Tax13-21, which lacks Leu at position 11. This may be attributable in part to the presence of another set of Leu and Val in the sequences at positions 11 and 17, respectively. These residues may also be able to act as the anchor residues into the MHC molecule that directs the antigenic residues toward the T-cell receptor. Tax177-186 did not include the epitope recognized by the Tax-specific CTL, although it possessed potential HLA-A2-specific residues. A synthetic peptide corresponding to the amino acid sequence 2-25 of HTLV-I Tax protein was also identified as an epitope of CTL with HLA-A2 restriction by other investigators (30). This is consistent with the results in the present study.

We previously demonstrated that the CTL established from HTLV-I carriers with HAM/TSP or Sjögren syndrome by in vitro stimulation with native HTLV-I antigens were cytotoxic predominantly to the target cells expressing HTLV-I Tax. These CTL also demonstrated less cytotoxicity to the targets expressing HTLV-I *env*, *gag*, or other pX gene products. A recent report demonstrated that Tax-specific CTL activity is readily detectable in PBL of HAM/TSP patients (15), although we detected such activity only after in vitro stimulation. This is probably due to differences in the stage of the disease. From these experiments, using recombinant vaccinia viruses, we learned that the cellular immune response of these patients against HTLV-I was directed especially to the Tax protein among HTLV-I antigens. However, it is still controversial whether Tax itself can be the target antigen of the CTL, since HTLV-I Tax is known to *trans*-activate expression of various cellular genes (12, 24, 29). In the present study, we identified a CTL epitope within HTLV-I Tax and clarified that the Tax protein itself is the target antigen recognized by the CTL. Furthermore, the competitor cells sensitized with the peptides including this epitope almost completely inhibited the cytotoxicity against the target cells infected with vaccinia virus-HTLV-I recombinants expressing Tax. This suggests that the CTL epitope identified here is the main one recognized by the HLA-A2-restricted Tax-specific CTL.

The relationship between HTLV-I infection and neural tissue damage in HAM/TSP is still unclear. T cells may attack neural cells infected with HTLV-I. However, no direct evidence of HTLV-I infection in neural cells has been shown so far, and neural cells could be resistant to the CTL because of the absence of MHC class I antigens (16). Merely the local reaction between cellular immunity and HTLV-I-infected lymphocytes may cause myelopathy via some factors without preceding HTLV-I infection in neural cells. Alternatively, HTLV-I-specific CTL may attack normal cells expressing self antigens similar to HTLV-I antigens. The elevation of HTLV-I antibodies in the cerebrospinal fluid suggests local HTLV-I infection. Transgenic experiments on mice transfected with HTLV-I pX and *env* gene demonstrated that the transfected gene was expressed in various tissues including brain, joint, salivary gland, muscle, and skin (10, 14), suggesting that these tissues potentially express HTLV-I antigens provided HTLV-I infects them. The distribution of these organs show a striking similarity to that of the organs affected by HTLV-I-associated disorders. The combination of HLA-A2 and the Tax epitope demonstrated here is one of the main target complexes recognized by HTLV-I-specific CTL derived from the patients with HAM/TSP or Sjögren syndrome tested. HAM/TSP and adult T-cell leukemia patients show some differences in human leukocyte antigen (HLA) haplotypes (27). Particular combinations of MHC molecules and HTLV-I antigen epitopes might induce a pathological T-cell response, although further investigation will be required to evaluate this. This possibility should be carefully considered in developing vaccines for

HTLV-I, because some epitopes might induce hazardous side effects in the host.

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