Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 4036–4040, April 1995
Medical Sciences

Differential activation of proliferation and cytotoxicity in human T-cell lymphotropic virus type I Tax-specific CD8 T cells by an altered peptide ligand

(p56^{lck}/unresponsiveness)

PER HÖLLSBERG*†, WIM E. J. WEBER*, FERNANDO DANGOND*, VANDHNA BATRA*, ALESSANDRO SETTE‡, AND DAVID A. HAFLER*

*Laboratory of Molecular Immunology, Center for Neurologic Diseases, Brigham & Women's Hospital and Harvard Medical School, Boston, MA 02115; and ‡Department of Immunochemistry, Cytel Corp., San Diego, CA 92121

Communicated by Robert C. Gallo, National Institutes of Health, Bethesda, MD, January 3, 1995 (received for review June 14, 1994)

ABSTRACT **Human T-cell leukemia virus type I (HTLV-I) gives rise to a neurologic disease known as HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Although the pathogenesis of the disease is unknown, the presence of a remarkably high frequency of Tax-specific, cytotoxic CD8 T cells may suggest a role of these cells in the development of HAM/TSP. Antigen-mediated signaling in a CD8 T-cell clone specific for the Tax(11–19) peptide of HTLV-I was studied using analog peptides substituted in their T-cell receptor contact residues defined by x-ray crystallographic data of the Tax(11–19) peptide in the groove of HLA-A2. CD8 T-cell stimulation with the wild-type peptide antigen led to activation of p56^{lck} kinase activity, interleukin 2 secretion, cytotoxicity, and clonal expansion. A Tax analog peptide with an alanine substitution of the T-cell receptor contact residue tyrosine-15 induced T-cell-mediated cytolysis without activation of interleukin 2 secretion or proliferation. Induction of p56^{lck} kinase activity correlated with T-cell-mediated cytotoxicity, whereas interleukin 2 secretion correlated with [^{3}H]thymidine incorporation and proliferation. Moreover, Tax peptide analogs that activated the tyrosine kinase activity of p56^{lck} could induce unresponsiveness to secondary stimulation with the wild-type peptide. These observations show that a single amino acid substitution in a T-cell receptor contact residue of Tax can differentially signal CD8 T cells and further demonstrate that primary activation has functional consequences for the secondary response of at least some Tax-specific CD8 T cells to HTLV-I-infected target cells.**

Human T-cell leukemia virus type I (HTLV-I), the first known human retrovirus (1), gives rise to a number of human diseases, most notably adult T-cell leukemia (1) and a neurologic disease known as HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (2, 3). HAM/TSP is characterized by inflammatory lesions in the spinal cord with a chronic progressive demyelination (4). Although the pathogenesis of the disease is unknown, a strikingly high frequency of CD8 T cells in the cerebrospinal fluid specific for the HTLV-I Tax protein suggests the involvement of antiviral cytotoxic CD8 T cells in the development of the disease (5).

Cytotoxic CD8 T cells recognize viral peptide antigens of typically 8–10 amino acids in the context of major histocompatibility complex class I (6). This results in ligation of the T-cell receptor (TCR) and initiation of signal transduction events leading to activation of the cytolytic pathway and clonal expansion of the CD8 T cell. Although the temporal events in the signal transduction cascade are still poorly understood, it is likely that p56^{lck} plays a major role in CD8 T-cell activation (7, 8). Peripheral T cells from p56^{lck}-knock-out mice are severely impaired in their antigen-specific T-cell signaling (9) and impaired signaling in mutant Jurkat T cells deficient in p56^{lck} expression can be restored by its reexpression (10).

Single amino acid substitutions in antigenic peptides can alter the outcome of T-cell receptor ligation in a qualitative way (11–20) and may thus provide insight into the separate signaling pathways after antigen-specific triggering. We have used this approach to analyze the CD8 response to the HTLV-I Tax(11–19) peptide antigen. In HLA-A2 positive patients with HAM/TSP the majority of CD8 T cells recognize the Tax peptide 11–19 (5, 21). This peptide fits the proposed anchor motif for HLA-A2 binding (22) and has recently been crystalized in the peptide groove of HLA-A2 (23). This has defined the structural basis for synthesizing peptide analogs substituted in their TCR contact residues.

Here we provide evidence for differential signaling in human CD8 T cells by a single amino acid substitution in a TCR contact residue of the peptide antigen and further demonstrate that primary activation has functional consequences for the secondary response of at least some Tax-specific CD8 T cells to HTLV-I-infected target cells.

MATERIALS AND METHODS

Generation of Epstein–Barr Virus (EBV)-Transformed B Cells. EBV-transformed B cells were generated from mononuclear cells (24, 25) depleted for T cells by anti-CD2 and goat anti-mouse antibodies conjugated to magnetic beads (Dynabeads, Dynal, Great Neck, NY). The non-T-cell fraction was incubated in the presence of 10% filtered supernatant from the EBV-producing cell line B95.8. An autologous EBV-transformed B-cell line, KS.B, was kindly provided by S. Jacobson (National Institutes of Health, Bethesda).

Cloning of Tax-Reactive Human CD8 T Cells. CD8 T cells were isolated from mononuclear cells (kindly provided by S. Jacobson) from an HLA-A2 positive patient with HAM/TSP by negative selection. Mononuclear cells were incubated with a cocktail of mononuclear antibodies against CD4 (OKT4), γ–δ TCR (TCRδ$_1$, a generous gift of M. Brenner, Boston), natural killer cells (NKH-1), and B cells (B1) followed by goat anti-mouse antibodies conjugated to magnetic beads. Negatively selected cells, 95% CD8 T cells as determined by immunofluorescent staining and flow cytometry analysis, were initially stimulated with Tax(11–19)-pulsed autologous EBV-transformed B cells and maintained in interleukin 2 (IL-2)-conditioned medium for 4.5 weeks. Cell lines reactive to

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: EBV, Epstein–Barr virus; HTLV-I, human T-cell leukemia virus type I; HAM/TSP, HTLV-I-associated myelopathy/tropical spastic paraparesis; IL-2, interleukin 2; TCR, T-cell receptor.

†To whom reprint requests should be addressed.

Tax(11–19) were single cell cloned with phytohemagglutinin (PHA) under limiting dilution conditions (24). T-cell clones were maintained by weekly restimulation with PHA and γ-irradiated mononuclear cells (24).

Primary Peptide Antigen Stimulation Assay. Synthetic tax peptides (Bio-Synthesis, Lewisville, TX) were >93% pure as determined by HPLC. To examine the CD8 T-cell response to the various Tax analog peptides, autologous EBV-transformed B cells (KS.B) were preincubated with peptide for 2 hr and then washed twice. One hundred microliters (10^5 per ml) of γ-irradiated (5000 rad; 1 rad = 0.01 Gy) KS.B cells was added to round-bottom 96-well plates (Costar) containing 100 μl of T cells (10^6 per ml). The cells were incubated in medium without IL-2 for 66 hr in the presence of 1 μCi of [^{3}H]thymidine per ml (1 Ci = 37 GBq) during the last 16 hr. Cells were harvested on a Tomtec cell harvester and counted in a betaplate counter (LKB, Wallac, 1205 Betaplate counter). Results are indicated as mean values of triplicates ± SE.

Secondary Peptide Antigen Stimulation Assay. Primary stimulation with peptide-pulsed KS.B cells was performed as described above but in the presence of IL-2 for 3 days. Cells were then washed and rested for another 4 days in complete medium with IL-2. Secondary proliferation was performed as described for the primary peptide antigen stimulation assay. When tested for secondary IL-2-induced proliferation, 5 units of recombinant IL-2 per ml (Boehringer Mannheim) was added instead of peptide-pulsed KS.B cells.

Cytotoxic Assay. Cytotoxicity was measured by a ^{51}Cr-release assay as described (25). Allogeneic or autologous EBV-transformed B cells were used as target cells in effector:target ratios of 0.25, 2.5, and 25. Specific release of ^{51}Cr was calculated as described (25).

Protein Tyrosine Kinase Assay. *In vitro* kinase assay on p56^{lck} was performed as described (26). In brief, Tax-reactive T cells (KS.2E11.7) were incubated with 10 μM peptide in the presence or absence of antigen-presenting cells for the indicated time, washed, and lysed in lysis buffer (1% Triton X-100/20 mM Tris/150 mM NaCl, pH 8.0) for 25 min. Lysates were precleared with a 10% *Staphylococcus aureus* cell suspension (Pansorbin, Calbiochem) before immunoprecipitation with anti-CD8 antibodies. After washing twice in lysis buffer, an *in vitro* kinase reaction was performed on the immunoprecipitate by adding 32 μl of kinase buffer {Hepes, 7 mg/ml; $MgCl_2$, 0.75 mg/ml; $MnCl_2$, 0.75 mg/ml (all from Sigma); and 0.6 mCi of [γ-^{32}P]ATP per ml (NEN)}. The reaction was stopped after 10 min of incubation at room temperature by adding 50 μl of 2× sample buffer. Samples were boiled and separated by 10% SDS/PAGE.

HLA-A2 Binding Assay. Binding of peptides to HLA-A2 was performed as described (27, 28). This assay is based on the inhibition of a standard peptide to detergent-solubilized HLA-A2.1 molecules. In brief, the standard peptide (FLPSDYFPSV) was iodinated to a specific activity of 4.5×10^5 Ci/mol at the tyrosine residue using the chloramine-T method. This is a TCR contact residue and iodination does not impair the major histocompatibility complex binding of the standard peptide (28). Approximately 5 nM of the standard peptide was incubated for 2 days at room temperature with various concentrations of the test peptide (1 nM to 100 μM), 10 nM HLA-A2.1 molecules, 1 mM phenylmethylsulfonyl fluoride, 1.3 mM 1,10-phenanthroline, 73 μM pepstatin A, 8 mM EDTA, 200 μM N^{α}-(*p*-tosyl)lysine chloromethyl ketone, and 0.05% Nonidet P-40. HLA-A2.1-bound radioactivity was determined by gel filtration and the concentration yielding 50% inhibition of the standard peptide was calculated (IC_{50}) as well as the test peptide/standard peptide binding ratio.

IL-2 ELISA. KS.2E11.7 was incubated with KS.B prepulsed with the indicated peptide as described for thymidine incorporation assay. After 40 hr of incubation, supernatants were collected and tested for the presence of IL-2 in an IL-2 ELISA kit (Endogen, Cambridge, MA). Samples were tested in duplicates and the amount of IL-2 was calculated from a standard curve and expressed in pg/ml. The sensitivity of the assay is 5 pg/ml according to the manufacturer.

RESULTS

Generation of Tax-Reactive CD8 T-Cell Clones. The Tax-reactive T-cell clone KS.2E11.7 was established from a Tax-reactive T-cell line by single cell cloning under limiting dilution conditions (24). KS.2E11.7 was $CD4^-CD8^+$ as determined by immunofluorescence and flow cytometry analysis and displayed specificity for the Tax(11–19) peptide. That is, KS.2E11.7 was cytolytic for autologous or HLA-A2-restricted allogeneic EBV-transformed B cells pulsed with 10 μM Tax(11–19) peptide but failed to lyse Tax(11–19)-pulsed major histocompatibility complex class I mismatched EBV-transformed B cells or HLA-A2-restricted targets that were not pulsed with the Tax(11–19) peptide (data not shown). KS.2E11.7 did not transcribe the HTLV-I Tax/Rex mRNA as determined by reverse transcription PCR but contained the HTLV-I long terminal repeat sequence in genomic DNA, suggesting that KS.2E11.7 was nonproductively infected by HTLV-I (data not shown).

Alanine Substitution of the TCR Contact Residues of the Immunodominant Tax(11–19) Peptide Does Not Affect Binding to HLA-A2. We designed Tax(11–19) peptide analogs substituted in the TCR contact residues based on the structural information from the crystallization of the Tax(11–19) peptide in the groove of HLA-A2 (23). These data indicated that the potential TCR contact residues are leucine in position 11 (11L), tyrosine in position 15 (15Y), proline in position 16 (16P), and tyrosine in position 18 (18Y). Analog peptides with alanine substitution of the TCR contact sites were tested for *in vitro* binding to HLA-A2 by a quantitative assay based on the inhibition of a radiolabeled standard peptide (FLPSDYFPSV) to detergent-solubilized HLA-A2 molecules (27). As shown in Table 1, the wild-type peptide and its analog peptides displayed good and virtually indistinguishable HLA-A2 binding capacity, confirming that these substitutions did not impair binding affinity. These peptides are thus expected to be presented to the TCR with approximately the same efficiency.

Separation of Proliferation and Cytolytic Function of Human CD8 T Cells by a Single Amino Acid Substitution in the Immunodominant Tax(11–19) Peptide. We next investigated whether the Tax analog peptides allowed for separation of the signals involved in clonal expansion and cytolytic function in the Tax-reactive CD8 T cells. Fig. 1 shows the ability of KS.2E11.7 to kill ^{51}Cr-labeled autologous EBV-transformed B cells (KS.B) prepulsed with Tax(11–19) or its analog peptides. Surprisingly, presentation of Tax(15Y→A) stimulated the cytolytic activity of the KS.2E11.7, although this peptide

Table 1. Alanine substitution of TCR contact residues in the Tax(11–19) peptide does not affect binding to HLA-A2

Peptide	Sequence†	HLA-A2 binding*	
		Relative	IC_{50}, nM
Tax(11–19)	LLFGYPVYV	1.3	3.8
Tax(11L→A)	**A**LFGYPVYV	0.7	7.4
Tax(15Y→A)	LLFG**A**PVYV	0.6	8.6
Tax(16P→A)	LLFGY**A**VYV	1.1	4.5
Tax(18Y→A)	LLFGYPV**A**V	1.1	4.5

*HLA-A2 binding was measured by a competitive assay based on the inhibition of binding of a radiolabeled standard peptide (FLPSDYFPSV) to detergent-solubilized major histocompatibility complex molecules. Results are expressed in relative binding (test peptide/standard peptide ratio) and as the concentration needed for 50% inhibition (IC_{50}).

†Alanine substitutions are indicated in boldface type.

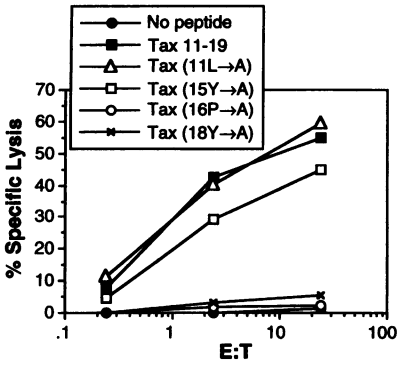

FIG. 1. Induction of cytotoxicity in CD8 T cells by Tax analog peptides. Percent specific lysis of ^{51}Cr-labeled autologous EBV B cells prepulsed with 33 μM Tax(11–19) peptide or alanine-substituted analogs by a Tax(11–19)-specific T-cell clone (KS.2E11.7). A representative of two independent experiments is shown. E:T, effector:target ratio.

analog was unable to trigger a proliferative response (Fig. 2). In contrast, Tax(11–19) and Tax(11L→A) induced a proliferative response and cytolytic function of KS.2E11.7, whereas Tax(16P→A) and Tax(18Y→A) were unable to trigger either proliferation or cytolysis (Figs. 1 and 2). Although other Tax-reactive T cells with different TCR sequences may respond differently, these data definitively demonstrated that a single amino acid change in an antigenic peptide allows for differentiation of the signaling events in human CD8 T cells leading to cytolysis and proliferation. Furthermore, three of the four substitutions in the Tax(11–19) peptide (15Y→A, 16P→A, and 18Y→A) failed to induce clonal expansion of KS.2E11.7 (Fig. 2). This may not be surprising, since amino acid changes in an antigenic peptide normally lower the affinity for the TCR (11).

The Tax(15Y→A) Analog Peptide Signals Through the TCR. The analog peptide Tax(15Y→A) was able to induce cytolysis but not proliferation, suggesting that this peptide allowed separation of distinct pathways leading to cytolysis and proliferation. Thus, we compared early and late signaling events in KS.2E11.7 upon stimulation with Tax(11–19) or its analog peptides. Given the potential importance of the protein tyrosine kinase p56^{lck} in antigen-specific activation of CD8 T cells (7–10), we examined its state of activation after stimulation with the wild-type Tax(11–19) peptide. *In vitro* autophosphorylation kinase assays on p56^{lck} coimmunoprecipitated with CD8 were performed on KS.2E11.7 incubated with KS.B in the presence of 10 μM Tax(11–19) peptide. As shown

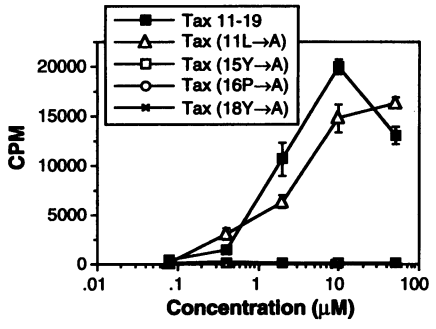

FIG. 2. Induction of [^{3}H]thymidine uptake in Tax-specific CD8 T cells by Tax analog peptides. KS.2E11.7 was stimulated with γ-irradiated, autologous EBV B cells (KS.B) prepulsed with the indicated peptides as in Fig. 1. Cells were incubated for 3 days and pulsed with [^{3}H]thymidine during the last 16 hr of incubation. Unpulsed KS.B did not stimulate [^{3}H]thymidine incorporation in KS.2E11.7 (110 cpm). A representative of three independent experiments is shown ± SE.

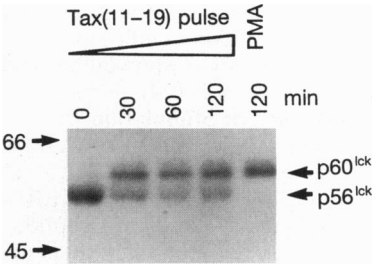

FIG. 3. Induction of protein tyrosine kinase p56^{lck} activation by antigen-specific crosslinking of the TCR in human CD8 T cells. The CD8 T-cell clone, KS.2E11.7 (8×10^6), and the autologous EBV B-cell line, KS.B (10^6) were incubated for a total of 2 hr in the absence or presence of 10 μM peptide for 30 min, 1 hr, and 2 hr, respectively. A separate sample was stimulated with 50 ng of phorbol 12-myristate 13-acetate (PMA) per ml for 2 hr as a positive control. After incubation the cells were lysed and immunoprecipitated with an anti-CD8 antibody. Protein tyrosine kinase activity was determined by an *in vitro* autophosphorylation assay. The autoradiograph was exposed for 30 min at room temperature. Molecular masses are indicated in kDa.

in Fig. 3, unstimulated T cells (lane 1) displayed a band of 56 kDa corresponding to p56^{lck}. Upon stimulation with the phorbol ester, phorbol 12-myristate 13-acetate (50 ng/ml), a slower migrating band appeared (lane 5) indicative of activated p56^{lck}, as has previously been reported (29). Importantly, however, stimulation of KS.2E11.7 with the Tax(11–19) peptide resulted in a retarded band of similar molecular mass (lanes 2–4), demonstrating that antigen-specific activation of human CD8 T cells results in p56^{lck} activation.

We subsequently determined whether the peptide analogs were able to induce an early event in T-cell activation such as protein tyrosine kinase p56^{lck} activity. As shown in Fig. 4, Tax(11–19), Tax(11L→A), and Tax(15Y→A) but not Tax(16P→A) or Tax(18Y→A) activated tyrosine kinase activity of CD8-associated p56^{lck}, providing definitive evidence for signal transduction induced by Tax(15Y→A). In contrast, presentation of Tax(15Y→A) was unable to induce a late event such as IL-2 production (Fig. 5), as opposed to Tax(11–19) and the Tax(11L→A) analog peptide. Thus, IL-2 production correlated with the ability to induce proliferation (compare Figs. 5 and 2), whereas the p56^{lck} activity correlated with the cytolytic function (compare Figs. 4 and 1).

Induction of Unresponsiveness in Human CD8 T Cells. Anergy in CD4 T cells may be induced by either lack of appropriate costimulation during antigen recognition (30) or stimulation with analog peptides (12). The requirements for induction of anergy in human CD8 T cells are less studied. We

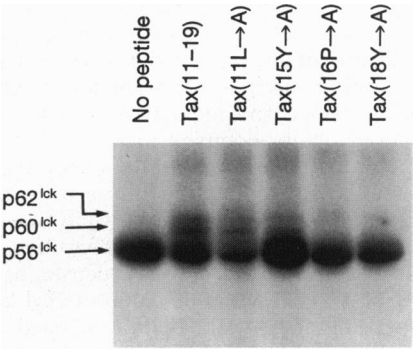

FIG. 4. Induction of early signaling events by Tax(15Y→A) in CD8 T cells. Tax-reactive CD8 T cells, KS.2E11.7 (10^7), were incubated for 1 hr in the presence of 20 μM Tax(11–19), analog peptide, or no peptide, as indicated. Cells were lysed, immunoprecipitated, and assayed for *in vitro* kinase activity as in Fig. 3. The autoradiograph was exposed for 20 min at room temperature.

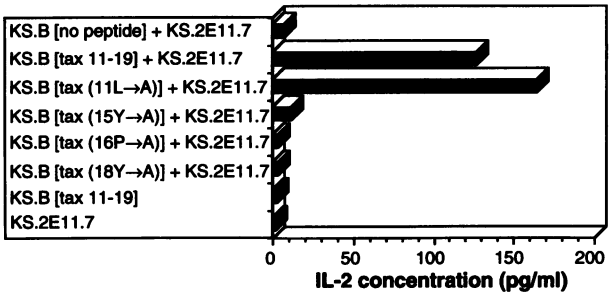

FIG. 5. Induction of IL-2 secretion by Tax analog peptides. Supernatants from the Tax-reactive CD8 T-cell clone, KS.2E11.7, stimulated for 40 hr with autologous EBV B cells (KS.B) pulsed with Tax(11–19) peptide or analog peptides were tested for the presence of IL-2 by ELISA. Supernatants from KS.2E11.7 alone or in the presence of unpulsed KS.B and KS.B pulsed with Tax(11–19) without KS.2E11.7 were included as controls.

hypothesized that the partial stimulation by Tax(15Y→A) might induce unresponsiveness to subsequent stimulation with the wild-type Tax(11–19) peptide in analogy to the CD4 model. To test this, we stimulated KS.2E11.7 with KS.B pulsed with the various peptides for 3 days, rested KS.2E11.7 in IL-2-conditioned medium for 4 days, and rechallenged them with a secondary stimulation with recombinant IL-2 (rIL-2) or KS.B pulsed with the various peptides, respectively. Table 2 shows that all peptides that were able to induce $p56^{lck}$ activity also induced unresponsiveness upon secondary stimulation. In particular, primary stimulation with Tax(15Y→A), which was unable to induce a proliferative response, rendered the KS.2E11.7 clone unresponsive to secondary stimulation with either Tax(11–19) or Tax(11L→A). This was in contrast to primary stimulation with no peptide, Tax(16P→A), or Tax(18Y→A) that allowed a secondary response after stimulation with Tax(11–19) or Tax(11L→A). Whereas the primary stimulation with Tax(11–19) rendered KS.2E11.7 antigen unresponsive, it did not alter the rIL-2-induced proliferation (data not shown). Similarly, KS.2E11.7 T cells rendered unresponsive by primary presentation of the Tax(15Y→A) peptide were still responsive to rIL-2. Although the mechanisms responsible for the unresponsiveness in this system have yet to be elucidated, these data further demonstrate that stimulation with the analog peptide Tax(15Y→A) has functional consequences for the CD8 T-cell activation.

DISCUSSION

Here we demonstrate that a hierarchy exists in the TCR-mediated signal transduction pathways, which allows antigen-specific induction of cytolysis without proliferation. Peptide analogs that activated the tyrosine kinase activity of $p56^{lck}$ induced unresponsiveness to a secondary stimulation with the wild-type peptide. Noticeably, the substituted peptide Tax(15Y→A) was unable to induce IL-2 secretion or proliferation but did induce $p56^{lck}$ tyrosine kinase activity and anergy in the Tax-reactive CD8 T cells. These results are summarized in Table 3.

Table 3. Summary of the functional response of a Tax-reactive CD8 T-cell clone to single amino acid substitutions in the TCR contact residues of the peptide antigen

Parameter	Tax peptide				
	Tax(11–19)	11L→A	15Y→A	16P→A	18Y→A
[^{3}H]Thymidine uptake	+	+	−	−	−
Cytotoxicity	+	+	+	−	−
IL-2 secretion	+	+	−	−	−
$p56^{lck}$ activity	+	+	+	−	−
Unresponsiveness	+	+	+	−	−

The direct demonstration of antigen-specific activation of $p56^{lck}$ adds to the existing evidence implicating $p56^{lck}$ as a critical tyrosine kinase in the TCR signaling pathway. In contrast to experiments with constitutively activated $p56^{lck}$ (31, 32), we did not observe IL-2 secretion after stimulation with the Tax(15Y→A) peptide, although this peptide activated $p56^{lck}$ activity. This could potentially be explained by transient activation of $p56^{lck}$ due to a negative-feedback mechanism that may not be operative on the constitutively activated F505 mutant of $p56^{lck}$ (8).

The Tax analog peptides used in this study segregated into three groups based on their effect on KS.2E11.7. The Tax(11L→A) peptide behaved as the wild-type Tax(11–19) peptide and induced cytolysis and proliferation. The Tax(11L→A) peptide induced slightly less thymidine incorporation than the wild-type Tax(11–19) peptide in the experiment shown in Fig. 2 and Table 2 and it is therefore possible that the 11L→A substitution slightly reduced the affinity for the TCR. The peptides Tax(16P→A) and Tax(18Y→A) induced neither cytolysis nor proliferation, and it is therefore possible that these peptides were significantly impaired in their TCR binding. Finally, the Tax(15Y→A) induced cytolysis but not proliferation. This differential T-cell activation indicated that Tax(15Y→A) failed to activate a pathway necessary for proliferation.

The analog peptides that were able to induce cytolysis and $p56^{lck}$ kinase activity also induced unresponsiveness or anergy in the KS.2E11.7. That is, 7 days after primary antigen stimulation with either the wild-type peptide or Tax(15Y→A), KS.2E11.7 was still unresponsive to secondary TCR-mediated antigen stimulation while remaining highly sensitive to IL-2 stimulation. This further demonstrated that the peptide Tax(15Y→A) was able to induce a signal although it was insufficient in triggering IL-2 secretion or a proliferative response in KS.2E11.7. Unresponsiveness was also induced if allogeneic HLA-A2-expressing EBV-transformed B cells were used as antigen-presenting cells (APCs). These APCs also expressed the restriction element for an MBP (peptide 84–102)-specific $CD4^+$ T-cell clone. However, in contrast to the Tax-specific CD8 T cells, anergy was not induced in the CD4

Table 2. Induction of unresponsiveness in HTLV-I Tax-specific CD8 T cells

Primary stimulation	Secondary stimulation					
	No peptide	Tax(11–19)	Tax(11L→A)	Tax(15Y→A)	Tax(16P→A)	Tax(18Y→A)
No peptide	115 ± 5	19,774 ± 912	10,750 ± 725	409 ± 30	128 ± 14	112 ± 14
Tax(11–19)	118 ± 20	124 ± 13	141 ± 21	126 ± 24	135 ± 26	135 ± 26
Tax(11L→A)	118 ± 35	99 ± 20	69 ± 8	101 ± 7	105 ± 14	105 ± 14
Tax(15Y→A)	116 ± 9	298 ± 60	119 ± 21	112 ± 16	125 ± 12	125 ± 12
Tax(16P→A)	90 ± 5	18,510 ± 500	11,149 ± 352	554 ± 34	95 ± 12	94 ± 5
Tax(18Y→A)	84 ± 8	22,952 ± 1376	13,602 ± 365	194 ± 13	94 ± 10	97 ± 9

The Tax-reactive CD8 T-cell clone KS.2E11.7 was stimulated with γ-irradiated, peptide-pulsed autologous EBV-transformed B cells (KS.B) for 3 days (primary stimulation), rested in IL-2-conditioned medium for 4 days, and rechallenged with a secondary stimulation with KS.B pulsed with the analog peptides, as indicated. Thymidine incorporation (cpm ± SE) was determined for the secondary stimulation.

T cells under these conditions (P.H. and D.A.H., unpublished data). When the secondary stimulation of anergic KS.2E11.7 was performed in the presence of IL-2 the T cells incorporated thymidine, indicating that they were not programmed to undergo cell death (P.H. and D.A.H., unpublished data). Furthermore, stimulation of KS.2E11.7 with the Tax analog peptides in the presence of IL-2 did not increase trypan blue uptake when compared to KS.2E11.7 treated with IL-2 alone.

As a model of CD8 T-cell activation we used a defined viral peptide that has been shown to be immunodominant in HLA-A2 positive patients with HAM/TSP (5, 33). If clonal expansion of Tax-reactive CD8 T cells is causally related to disease development in HAM/TSP patients, identification of peptides that are able to anergize these CD8 T cells may be of therapeutic use.

We thank Scott Southwood for technical assistance and Dr. Steven Jacobson for the generous gift of mononuclear cells and EBV-transformed B cells from an HLA-A2 positive HAM/TSP patient. This work was supported by grants from the National Multiple Sclerosis Society (P.H., W.E.J.W., and D.A.H.), Stichting Vrienden Multiple Sclerosis Research (W.E.J.W.), a Howard Hughes Medical Institute Postdoctoral Fellowship for physicians (F.D.), and National Institutes of Health Grants AI18634 (A.S.) and NS24247 (D.A.H.).

1. Poiesz, B. J., Ruscetti, F. W., Gazdar, A. F., Bunn, P. A., Minna, J. D. & Gallo, R. C. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 7415–7419.
2. Gessain, A., Barin, F., Vernant, J. C., Gout, O., Maurs, L., Calender, A. & de Thé, G. (1985) *Lancet* **ii,** 407–410.
3. Osame, M., Izumo, S., Igata, M., Matsumoto, T., Matsumoto, S., Sonoda, M., Tara, M. & Shibata, Y. (1986) *Lancet* **ii,** 104–105.
4. Höllsberg, P. & Hafler, D. A. (1993) *N. Engl. J. Med.* **328,** 1173–1182.
5. Elovaara, I., Koenig, S., Brewah, Y., Woods, R. M., Lehky, T. & Jacobson, S. (1993) *J. Exp. Med.* **177,** 1567–1573.
6. Germain, R. N. (1994) *Cell* **76,** 287–299.
7. Rudd, C. E., Janssen, O., Prasad, K. V. S., Raab, M., da Silva, A., Telfer, J. C. & Yamamoto, M. (1993) *Biochim. Biophys. Acta* **1155,** 239–266.
8. Chan, A. C., Desai, D. M. & Weiss, A. (1994) *Annu. Rev. Immunol.* **12,** 555–592.
9. Molina, T. J., Kishihara, K., Siderovski, D. P., van Ewijk, W., Narendran, A., Timms, E., Wakeham, A., Paige, C. J., Hartmann, K. U., Veilette, A., Davidson, D. & Mak, T. W. (1992) *Nature (London)* **357,** 161–164.
10. Straus, D. & Weiss, A. (1992) *Cell* **70,** 585–593.
11. Evavold, B. D., Sloan-Lancaster, J. & Allen, P. M. (1993) *Immunol. Today* **14,** 602–609.
12. Evavold, B. D. & Allen, P. M. (1991) *Science* **252,** 1308–1310.
13. De Magistris, M. T., Alexander, J., Coggeshall, M., Altman, A., Gaeta, F. C. A., Grey, H. M. & Sette, A. (1992) *Cell* **68,** 625–634.
14. Racioppi, L., Ronchese, F., Matis, L. A. & Germain, R. N. (1993) *J. Exp. Med.* **177,** 1047–1060.
15. Evavold, B. D., Sloan-Lancaster, J., Hsu, B. L. & Allen, P. (1993) *J. Immunol.* **150,** 3131–3140.
16. Jameson, S. C., Carbone, F. R. & Bevan, M. J. (1993) *J. Exp. Med.* **177,** 1541–1550.
17. Sloan-Lancaster, J., Evavold, B. D. & Allen, P. M. (1993) *Nature (London)* **363,** 156–159.
18. Alexander, J., Snoke, K., Ruppert, J., Sidney, J., Wall, M., Southwood, S., Oseroff, C., Arrhenius, T., Gaeta, F. C. A., Colón, S. M., Grey, H. M. & Sette, A. (1993) *J. Immunol.* **150,** 1–7.
19. Ruppert, J., Alexander, J., Snoke, K., Coggeshall, M., Herbert, E., McKenzie, D., Grey, H. M. & Sette, A. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 2671–2675.
20. Sette, A., Alexander, J., Ruppert, J., Snoke, K., Franco, A., Ishioka, G. & Grey, H. M. (1994) *Annu. Rev. Immunol.* **12,** 413–431.
21. Jacobson, S., Shida, H., McFarlin, D. E., Fauci, A. S. & Koenig, S. (1990) *Nature (London)* **348,** 245–248.
22. Falk, K., Rötzschke, O., Stevanovic, S., Jung, G. & Rammensee, H. G. (1994) *Nature (London)* **351,** 290–296.
23. Madden, D. R., Garboczi, D. N. & Wiley, D. (1993) *Cell* **75,** 693–708.
24. Höllsberg, P., Wucherpfennig, K. W., Ausubel, L. J., Calvo, V., Bierer, B. E. & Hafler, D. A. (1992) *J. Immunol.* **148,** 3256–3263.
25. Weber, W. E. J., Buurman, W. A., Vandermeeren, M. M. P. P. & Raus, J. C. M. (1985) *J. Immunol.* **135,** 2337–2342.
26. Rudd, C. E., Trevillyan, J. M., DasGupta, J. D., Wong, L. L. & Schlossman, S. F. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 5190–5194.
27. Ruppert, J., Sidney, J., Celis, E., Kubo, R. T., Grey, H. M. & Sette, A. (1993) *Cell* **74,** 929–937.
28. Sette, A., Sidney, J., Delguercio, M. F., Southwood, S., Ruppert, J., Dahlberg, C., Grey, H. M. & Kubo, R. T. (1994) *Mol. Immunol.* **31,** 813–822.
29. Veilette, A., Horak, I. D. & Bolen, J. B. (1988) *Oncogene Res.* **2,** 385–401.
30. Mueller, D. L., Jenkins, M. K. & Schwartz, R. H. (1989) *Annu. Rev. Immunol.* **7,** 445–480.
31. Caron, L., Abraham, N., Pawson, T. & Veilette, A. (1992) *Mol. Cell. Biol.* **12,** 2720–2729.
32. Luo, K. & Sefton, B. M. (1992) *Mol. Cell. Biol.* **12,** 4724–4732.
33. Koenig, S., Woods, R. M., Brewah, Y. A., Newell, A. J., Jones, G. M., Boone, E., Adelsberger, J. W., Baseler, M. W., Robinson, S. M. & Jacobson, S. (1993) *J. Immunol.* **156,** 3874–3883.