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High Activated and Memory Cytotoxic T-cell Responses to HTLV-1 in Healthy Carriers and Patients with Tropical Spastic Paraparesis

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The cytotoxic T-lymphocyte (CTL) response to HTLV-1 is directed mainly against the Tax protein. Circulating, activated Tax-specific CTL can be found in a majority of healthy carriers and patients with the HTLV-1-associated disease tropical spastic paraparesis (HAM/TSP). In this study we present data on the Tax-specific CTL response of 26 HTLV-1 carriers, including 10 newly recruited subjects. Rex-specific CTL were not found in any subjects investigated. Activated and memory CTL responses were determined separately in 4 healthy carriers, 3 HAM/TSP patients, and 1 "seronegative HAM/TSP." In all subjects, the mean frequency of peptide-specific memory cells per epitope (1/1307) was high. There was no significant difference in mean memory CTL frequency per epitope or in the proportion of subjects with activated CTL between healthy carriers and HAM/TSP patients. One individual with HAM/TSP had an unusually high frequency response to two peptides, suggesting immunodominance of epitope recognition in this individual. We conclude that the magnitude and components of the HTLV-1-specific CTL response do not differ between healthy carriers and HAM/TSP patients. These data do not support a specific CTL-mediated component in the pathogenesis of HAM/TSP.

INTRODUCTION

Class I MHC-restricted cytotoxic T-cells (CTL) are potent anti-viral effectors *in vitro* and *in vivo* (Koup, 1994). $CD8^+$ CTL are frequently found in the peripheral blood of people infected with human T-cell leukemia virus type 1 (HTLV-1) (Jacobson *et al.,* 1990; Parker *et al.,* 1992). The CTL predominantly recognize epitopes in the Tax protein of HTLV-1 and in other viral proteins to a lesser extent (Parker *et al.,* 1994; Furukawa *et al.,* 1994).

The role (if any) of these immune cells in the pathogenesis of HTLV-1-associated diseases is not understood. Elovaara *et al.* (1993) measured the precursor frequencies of peripheral blood CTL and CSF-derived CTL in asymptomatic carriers and HAM/TSP patients. In peripheral blood lymphocytes, they reported a precursor frequency of Tax-specific CTL at least 40-fold higher in HAM/TSP patients than in asymptomatic HTLV-1 carriers and suggested that the presence of HTLV-1-specific CTL might contribute to the pathogenesis of HAM/TSP. We have found strong fresh CTL responses in asymptomatic HTLV-1-infected individuals as well as in HAM/TSP patients (Parker *et al.,* 1992, 1994), and to date we have been unable to identify a qualitative or quantitative difference in these responses between patient groups.

The Tax protein is persistently expressed in infected individuals and represents by far the most important target of CTL recognition in HTLV-1-infected individuals (Parker *et al.,* 1994). Tax is a regulatory protein which indirectly controls viral transcription via sequences in the viral long terminal repeat. Tax also induces the expression of several cellular proteins including cellular adhesion molecules. Several regions within Tax can be recognized by CTL simultaneously (Parker *et al.,* 1994) and there is evidence that certain epitopes are immunodominant.

Rex is the other major viral regulatory protein of HTLV-1: it is expressed from the same mRNA as Tax but in a different translational open reading frame. Rex also controls the expression of viral proteins by favoring translation from unspliced or singly spliced mRNAs and is thought to shuttle mRNA from the nucleus into the cytoplasm for translation to occur (Adachi *et al.,* 1993). There is no evidence of chronic Rex protein expression in HTLV-1 infection and a CTL response to Rex has not been described.

The two principal aims of the present study were (1) to compare the activated and memory CTL responses to HTLV-1 peptides in the peripheral blood $CD8^+$ T-cells between healthy carriers and HAM/TSP patients and (2) to look for a CTL response to Rex in both freshly isolated and cultured T-cells.

MATERIALS AND METHODS

Subjects. A total of 14 HTLV-1-seropositive, asymptomatic patients were investigated. Eight of these patients

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have been described elsewhere (Parker *et al.,* 1992, 1994), and 6 were newly recruited. In addition, 9 seropositive HAM/TSP patients were included in the study (5 have been described previously (Parker *et al.,* 1992, 1994), 4 were newly recruited). Subject TZ is a 51-year-old male born in St. Lucia who developed HAM/TSP and uveitis within the past 2 years. He was treated with 500 mg azidothymidine twice daily and 3 million units of interferon-alpha by subcutaneous injection daily for 6 weeks. Three blood samples from TZ were tested; the first during this treatment and the second and third, 4 days and 1 month after its discontinuation, respectively. Patients TAA and TW were undergoing treatment with 200 mg danazol (an anabolic steroid) three times daily at the time of being tested. Two patients with a clinical presentation consistent with HAM/TSP, but who are seronegative for HTLV-1, were also investigated. A third seronegative HAM/TSP patient has been described in detail before (Daenke *et al.,* 1994). In the subject codes, the prefix T refers to the presence of HAM/TSP, and H denotes a healthy carrier. The MHC class I allotype was determined for each patient by polymerase chain reaction (Krausa *et al.,* 1993) and, in some cases, by complement-mediated lysis using allospecific antisera.

CTL assays. CTL assays were standard 5-hr ^{51}Cr-release assays as described (Parker *et al.,* 1992). $CD8^+$ effector cells were isolated freshly from PBL by positive selection on M-450 anti-CD8-coated Dynabeads (Dynal). Following selection, beads were removed from the cells by incubation with anti-sheep IgG (Detachabead, Dynal). In fresh assays, unstimulated $CD8^+$ cells were tested within 24 hr of venesection. HTLV-1-specific lysis was determined using a one-tailed *t* test ($P < 0.05$) comparing peptide-pulsed or vaccinia-infected target cells with untreated target cells or targets pulsed with an irrelevant peptide.

Autologous EBV-transformed B-cell lines were used as target cells in cytotoxic T-cell assays. Targets were labeled with chromium and prepulsed with 50 μM peptide before being added to the effector cells. The concentration of 50 μM peptide was used in these assays in order that each peptide of a pool would not be limiting. Where single peptides are used to pulse target cells, this concentration can be reduced to 0.5 μM (data not shown). This method of target preparation has been described in detail elsewhere (Parker *et al.,* 1994). Percentage specific lysis was calculated as $100 \times$ [(test cpm $-$ background cpm)/(maximal cpm $-$ background cpm)].

CTL lines. T-cell lines were established *in vitro* without the addition of exogenous antigen, using $CD8^+$ cells cultured with equal numbers of autologous irradiated CD8-depleted feeder cells. Lines were expanded with 1 μg/ml phytohaemagglutinin (PHA) and 10% Lymphocult-T (Biotest Diagnostics) in RPMI 1640/10% fetal calf serum (FCS) and maintained for several weeks in RPMI/10% FCS/10% Lymphocult-T. For antigen-stimulated T-cell lines, unfractionated PBL were cultured with the addition of 200 μM pools of 3–10 peptides on Day 1, and cells were fed with Lymphocult-T as before. This reduced the peptide concentration to 50 μM over 7–10 days. These peptide-stimulated cultures were tested for CTL activity after 10–14 days of stimulation. This method has been shown to restimulate CTL memory cell responses in HIV-1 patients (Klenerman *et al.,* 1994) and HTLV-1 patients (S. Hall and C. Bangham, unpublished data).

Limiting dilution assays. Freshly selected $CD8^+$ lymphocytes were plated into 96-well plates at 200, 500, 1000, and 2500 cells per well, in 30 replicate wells for each cell input number. Autologous, irradiated CD8-depleted peripheral blood mononuclear "feeder" cells (10^5) were added to each well. Three days later, cells were fed with 1 μg/ml PHA in RPMI/10% FCS/10% Lymphocult-T. After approximately 10 days, cultures were split into replicate plates and fed with RPMI/10% FCS/10% Lymphocult-T. Chromium release assays were done using 3000 peptide-pulsed or untreated chromium-labeled autologous targets per well on Days 14–25 of culture. Frequency analysis was done by counting the number of replicate wells at each cell input number that showed specific cytotoxic lysis of target cells. The criterion of positivity in each assay was determined by calculating the mean specific lysis of the 30 replicates assayed on targets without peptide preincubation, +3 standard deviations of the mean. The least squares regression line was used to calculate a precursor frequency using the method of Lefkovits and Waldmann (1979). Frequencies are expressed as a fraction of $CD8^+$ cells isolated from peripheral blood.

Peptides. 15-Mer peptides spanning the entire protein sequences of Tax and Rex and overlapping by five amino acids were synthesised by F-moc chemistry. Peptides were grouped into pools of 10 for screening, and later 4, 3, or 2 peptides were pooled for mapping new epitopes. A peptide epitope from HTLV-1 reverse transcriptase (PC4) has been described before (Parker *et al.,* 1992).

RESULTS

CTL epitopes in Tax are evenly distributed throughout the protein

In previous work (Parker *et al.,* 1994) we identified nine CTL peptide epitopes in Tax, all of which map to the N-terminal half of the protein. We have extended this work by testing CTL responses in 10 newly acquired subjects and in several subjects who have been tested previously. In all cases, $CD8^+$ lymphocytes derived from these subjects were tested for responses against a number of peptide pools spanning the whole of the Tax or Rex proteins. We (Parker *et al.,* 1992, 1994) and others (Jacobson *et al.,* 1990; Furukawa *et al.,* 1994) have found Tax to be highly immunodominant as a CTL target antigen. In this study using peptide pools, we were able to identify up

Bar No.	Peptide	HLA allele	Responders	Bar No.	Peptide	HLA allele	Responders
1	XN2	nd	TZ	10	X3	B35	TZ
2	TVO	A2	HH, HM, TAA,TE, TY	11	X4	A2	HM, HY, TE
3	XN3	A2	HD, HH, HM, TE	12	X5	nd	TV
4	XN4	A2	HH, HM, TAA, TE	13	X6	nd	TV
5	XN7	B15	TE	14	X7	B7	HM, TU
6	XN11	A2	HD, HM, TE, HS	15	X10	B35	TZ
7	XN12	A2	HM, TE	16	X13	nd	HW
8	X1	nd	HT, HZ, TD	17	X19	B35	TZ
9	X2	B35	TZ	18	X20	B35	TZ

Boxed bar numbers denote newly identified epitopes in Tax.

FIG. 1. The full-length HTLV-1 Tax amino acid sequence is shown. Numbered bars above the sequence represent peptides recognized by CTL from HTLV-1-infected patients. Boxed bar numbers denote newly identified Tax epitopes. The reference name (as appears in the text) of each peptide and the HLA restriction element for recognition by CTL (where identified) is shown in the accompanying table. Subjects shown to respond to each peptide in fresh assays or in T-cell lines are indicated. Subjects HS, HT, HW, HX, HY, HZ, TAA, TV, TW, TX, TY, and TZ are patients newly recruited for this study.

to eight new CTL epitopes in Tax (Fig. 1). For most of these epitopes, the HLA restriction allele has been identified (Fig. 1) using target B-cell lines matched and mismatched at the HLA-A and HLA-B loci. The complete panel of Tax epitopes are spread throughout the protein sequence including the C-terminal end and are not clustered in the N-terminal half of the protein. In those cases where CTL responses to overlapping 15-mer peptides have been identified, effector frequency data show that these are in fact separate epitopes with different effector frequencies, although some are presented by the same MHC class I molecule (compare peptides X19, X20; Table 3). These data are therefore not consistent with a single epitope spanning the adjacent 15-residue peptides. While 15-mer peptides may be recognized with lower efficiency compared with shorter "optimized" peptides, our experience has been that, following clonal expansion in the limiting dilution assay, very few wells give marginal positive or negative cytolytic values. In addition, the magnitude of positivity does not alter the calculation of effector frequency (Lefkovits and Waldmann, 1979). We are confident that 15-mer peptides accurately estimate, if not closely underestimate, the effector frequencies of CTL. In all cases, Tax-specific lysis identified on peptide-pulsed targets was confirmed using endogenously presented Tax protein expressed from pX-recombinant vaccinia virus infected target cells (data not shown; Parker *et al.,* 1992).

HTLV-1 carriers with and without HAM/TSP have activated CTL to Tax

The data in Table 1 show CTL responses to Tax in individuals from each of two clinical groups. Of 14 seropositive healthy carriers studied, 10 had a fresh CTL response to Tax; in every case anti-Tax CTL were also found in a T-cell line derived from the $CD8^+$ cell population. Four of the healthy subjects did not produce a fresh response *in vitro* (HC, HG, HS, HX). Two of these were also negative when tested as a $CD8^+$ T-cell line (HC, HX), but HS was shown to recognize peptide XN11 after culture. A T-cell line was not established from HG.

In the HAM/TSP clinical group, of nine HTLV-1 seropositive HAM/TSP patients tested, five had a fresh response to Tax, four failed to recognize peptide-pulsed targets in a fresh assay (Table 1). In all of these patients except TB, HTLV-1 peptides were specifically recognized by their respective cultured T-cell lines. The proportion

TABLE 1

Tax-Specific CTL Responses in HTLV-1-Infected Subjects

Healthy subjects		Tax response		TSP subjects		Tax response	
Subject code	Antibody	Fresh	T-cell line	Subject code	Antibody	Fresh	T-cell line
HA	+	+	+	TB	+	+	nd
HB	+	+	+	TD	+	+	+
HD	+	+	+	TE	+	+	+
HH	+	+	+	TF	+	+	+
HM	+	+	+	TZ	+	+	+
HN	+	+	+	.(5)			
HT	+	+	+				
HW	+	+	+	TAA	+	Negative	+
HY	+	+	+	TI	+	Negative	+
HZ	+	+	+	TV	+	Negative	+
.(10)				TW	+	Negative	+
				.(4)			
HC	+	Negative	Negative				
HG	+	Negative	nd	TO	Sero-ve	Negative	nd
HS	+	Negative	+	TX	Sero-ve	Negative	Negative
HX	+	Negative	Negative	TY	Sero-ve	Negative	Negative[a]
.(4)				.(3)			

Note. $CD8^+$ cells from 14 healthy HTLV-1 carriers and 12 HAM/TSP patients were tested for recognition of HTLV-1 peptide antigens presented by autologous B-cell lines in a chromium release assay. The HTLV-1-specific lysis was called positive if it significantly ($P < 0.05$) exceeded the lysis of untreated target cells (Student's *t* test, one-tailed). A summary of these data for fresh CTL responses and T-cell line responses are shown. The HTLV-1-specific antibody status of each subject is shown. TO, TX, and TY were seronegative for HTLV-1 but were shown to have HTLV-1 proviral sequences in the genomic DNA. These patients had a clinical presentation consistent with HAM/TSP. nd, not done.

[a] TY did not recognize HTLV-1 Tax or Rex peptides in a fresh assay or in a bulk T-cell line but did show reactivity to peptide TVO (see Fig. 1) in the limiting dilution analysis.

of fresh CTL responders to Tax in the healthy carrier group (10/14) and the HAM/TSP group (5/9) did not differ significantly ($\chi^2 = 0.821$; $P > 0.25$ at 1 *df*).

Three seronegative patients in whom HTLV-1 proviral sequences have been identified (TO (Daenke *et al.,* 1994); TX, TY (M. Sommerlund and C. Bangham, unpublished data)), and who have a clinical syndrome indistinguishable from HAM/TSP, were included in the study. No CTL could be identified in subject TO who has been shown to have a deleted provirus integrated into his genomic DNA (Daenke *et al.,* 1994). Two newly recruited subjects, TX and TY likewise had no fresh CTL response to Tax peptides and no significantly detectable response to Tax in the T-cell lines established from their cells. However, in one case (TY), Tax-specific CTL were identified in a limiting dilution assay after expansion of the clones (see Table 3). This shows that weak CTL responses may not be detectable in a fresh assay of circulating cells, but HTLV-1-specific memory cells can be amplified by culture *in vitro* with PHA and IL-2.

Rex-specific CTL were not detected in any HTLV-1 infected subjects

Fourteen subjects were investigated for fresh CTL responses to Rex (Table 2). Thirteen of these subjects had been found to have Tax-specific CTL; 11 had fresh responses to Tax and 2 had responses in the T-cell line only. HW and TZ had Pol-specific CTL in addition. Subject HX was negative for a Tax-specific response but positive for HTLV-1 antibodies (see Table 1). Cytotoxic T-cell responses to Rex could not be found in any of these subjects. Assays for Rex responsiveness were done using peptide pools in all subjects. In all fresh assays, the effectors were tested within 12 to 24 hr of venesection, as in the assays for Tax-specific CTL. Where T-cell lines were established from the sample, these were tested 10–15 days after antigen nonspecific (PHA and Lymphocult-T) or antigen-specific (Rex peptide pools) stimulation *in vitro.* In 24 experiments, T-cell lines were tested for reactivity to Rex peptides one to three times without peptide stimulation and two to three times after peptide stimulation, but were consistently negative (data not shown).

Frequencies of HTLV-1 specific memory CTL are similar in healthy carriers and HAM/TSP patients

Frequencies of CTL in the peripheral blood of subjects from both clinical groups were studied by limiting dilution analysis. Both $CD4^+$ and $CD8^+$ T-cells from HTLV-1-infected subjects tend to proliferate spontaneously *in vitro* (Hollsberg *et al.,* 1992). In our analysis of these data, we have used the term "effector frequency" in preference to "precursor frequency" because, although it is likely that

TABLE 2

Rex-Specific CTL Responses in HTLV-1–Infected Subjects

Healthy subjects		Rex response		TSP subjects		Rex response	
Subject	Antibody	Fresh	T-cell line	Subject	Antibody	Fresh	T-cell line
HA	+	Negative	nd	TAA	+	Negative	nd
HB	+	Negative	nd	TD	+	Negative	nd
HM	+	Negative	Negative	TV	+	Negative	nd
HN	+	nd	Negative	TW	+	Negative	Negative
HT	+	Negative	Negative	TZ	+	Negative	nd
HW	+	nd	Negative				
HX	+	nd	Negative				
HY	+	Negative	nd				
HZ	+	Negative	Negative				

Note. Thirteen HTLV-1 Tax responsive patients (eight healthy; five HAM/TSP) were tested for CTL recognition of Rex peptides by fresh or cultured CTL. Subject HX (negative for Tax-specific CTL) was also tested. No Rex-specific CTL were detected in these subjects.

the majority of this response is due to restimulated memory CTL, some contribution of proliferating activated CTL cannot be excluded.

Limiting dilution cultures were assayed on peptide-pulsed autologous targets in all cases, after peptide epitopes had been identified in fresh CTL assays or T-cell lines. This allows efficient quantification of target-specific effectors because it does not depend on the efficiency of expression and processing of an endogenously produced recombinant protein. The calculated effector frequencies for each peptide epitope specified are shown in Table 3. The mean frequency of HTLV-1 peptide-specific effectors per epitope did not differ significantly between the healthy carriers and HAM/TSP patients (Student's *t* test; $t = -0.964$, $P = 0.358$ at 10 *df*).

In subject HM, frequencies of CTL effectors to two separate peptide epitopes were calculated. For these two epitopes, both of which are recognized by fresh CTL, frequency values were similar at 1/2643 $CD8^+$ (XN3) and 1/1392 $CD8^+$ cells (XN11). In subject TZ, five peptides were tested in the limiting dilution assay. Calculated frequency values ranged from 1/223 to 1/4218 $CD8^+$ cells (Table 3 and Fig. 2). These data show that some peptide-specific CTL are more frequent in the peripheral circulation than others in this individual. The total Tax-specific CTL effector frequency (sum of the frequencies for each epitope recognized simultaneously) in this subject was 1/129. The two peptides recognized by the highest frequencies of CTL in the limiting dilution analysis also gave the highest levels of lysis in assays of freshly isolated

TABLE 3

Frequencies of HTLV-1-Specific CTL in Healthy Carriers and TSP Patients

Subject	Target peptide	Frequency	Subject	Target peptide	Frequency
HM	XN3	1/2643	TD	PC4	1/2719
HM	XN11	1/1392	TV	X6	1/2618
HW	X13	1/3103	TZ	X2	1/3181
HZ	PC4	1/3197	TZ	X19	1/223
HS	XN11	1/3141	TZ	X20	1/4218
			TZ	X3	1/588
			TZ	X10	1/4176
Mean H freq/epitope		1/2438	Mean T freq/epitope		1/906
U1	PC4	1/94,870	TY	TV0	1/5838
U2	TV0	1/56,989			
Mean U freq/epitope		1/71,205			

Note. Effector frequencies from limiting dilution analysis of CTL responding to known peptide epitopes were calculated according to the method of Lefkovits and Waldmann (1979). For each epitope assayed, effector frequencies are represented as a fraction of the $CD8^+$ lymphocyte population (1/*x*, 1/*y*, etc.) positively selected from the peripheral blood of patients. The mean frequency values per epitope were calculated as the combined effector frequency to several epitopes ((1/*x*) + (1/*y*) + (1/*z*), etc)/n. Clinical groups are defined as healthy carriers (H), HAM/TSP patients (T), and uninfected controls (U).

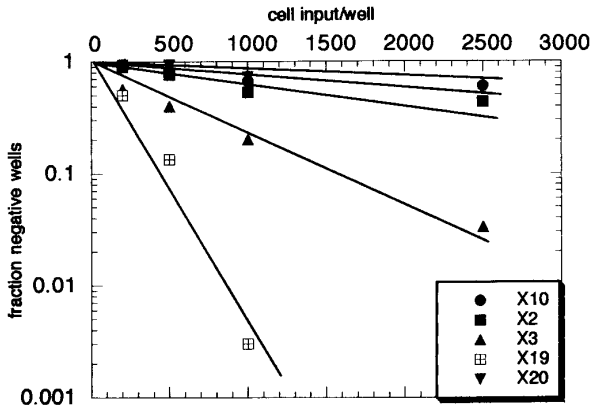

FIG. 2. Limiting dilution analysis of CTL from subject TZ. A semilog plot of the fraction of 30 replicate wells of CTL responding to peptide-pulsed autologous targets (*y*-axis) is shown as a function of the effector cell input/well (*x*-axis). Linear regression analysis allows the calculation of effector frequency which corresponds to the cell input number at which the fraction of unresponsive wells is 0.37. The figure shows the data for lysis of peptide targets X2, X3, X10, X19, and X20 by CTL from TZ. The frequency values calculated from these data appear in Table 3.

$CD8^+$ T-cells and the bulk culture of T-cells (compare Table 3 with Fig. 3). These results show that the peptides X3 and X19 were immunodominant in this subject.

A frequency of 1/5838 $CD8^+$ Tax-specific CTL was measured for subject TY. This HLA-$A2^+$, seronegative individual failed to show a CTL response to the HLA-A2-restricted Tax peptide 11–19 (LFGYPVYV) either in fresh assays or in nonantigen-stimulated T-cell lines. The frequency of effectors was lower than those seen in other individuals and may have fallen below the threshold of sensitivity of a bulk cytotoxic lysis assay. $CD8^+$ lymphocytes from the peripheral blood of two uninfected volunteers of known HLA type were assayed on autologous target B-cell lines pulsed with an appropriate HTLV-1-peptide. The effector frequencies were calculated as 1/56,989 and 1/94,870 $CD8^+$ cells in these subjects (Table 3). Although some positive wells were found in the limiting dilution assays, these did not titrate with cell input number and were similar to the frequencies calculated for recognition of untreated B-cell lines.

DISCUSSION

The presence of $CD8^+$ CTL specific for HTLV-1 antigens has been reported in healthy HTLV-1 carriers and in those with HAM/TSP. These CTL are predominantly directed to the Tax protein, but other viral gene products are also recognized on infected cells (Jacobson *et al.,* 1990; Parker *et al.,* 1992). HTLV-1-specific CTL have been found in the CSF and in peripheral blood (Elovaara *et al.,* 1993) and it has been suggested that they play a role in the pathogenesis of HAM/TSP by mounting a cytolytic attack against HTLV-1-infected cells in the CNS.

This study extends by 10 the number of individuals in whom we have identified $CD8^+$ anti-HTLV-1 responses. Eleven of 26 subjects (5 healthy carriers and 6 HAM/TSP patients) respond to more than one epitope in Tax (see Fig. 1). Fresh CTL responses were found in a similar proportion of subjects belonging to each clinical group as were CTL responses in T-cell lines derived from peripheral $CD8^+$ cells. This shows that HTLV-1-infected healthy carriers and HAM/TSP patients are equally able to mount a CTL response to the virus and that Tax expression is persistent in most infected individuals.

HAM/TSP patients have been shown to have a higher viral load than healthy carriers (Yoshida *et al.,* 1989; Shinzato *et al.,* 1993). Recently, however, the levels of tax mRNA per infected cell in healthy carriers and HAM/TSP patients has been shown to be the same (Furukawa *et al.,* 1995). This suggests that the differences in viral load and levels of viral expression between healthy and HAM/TSP patients relate to the number of infected cells per patient rather than the transcriptional activity of the virus. This might suggest that healthy carriers have a more effective CTL response to HTLV-1 infection than HAM/TSP patients.

Elovaara *et al.* (1993) found that HAM/TSP patients had a higher frequency of CTL responder cells circulating in the peripheral blood than did healthy patients. However, these data compared recognition of peptide-pulsed targets (presentation of a single epitope) with vaccinia-pX-infected targets (presentation of single or multiple epitopes) and are difficult to interpret. We were unable to reproduce these findings in our group of subjects using peptide-pulsed targets in all assays. We found no differences in the effector CTL frequencies between healthy carriers and HAM/TSP patients, although in one individual, we showed clear immunodominance of CTL reactivity to two Tax peptides. In this individual (TZ), the effector

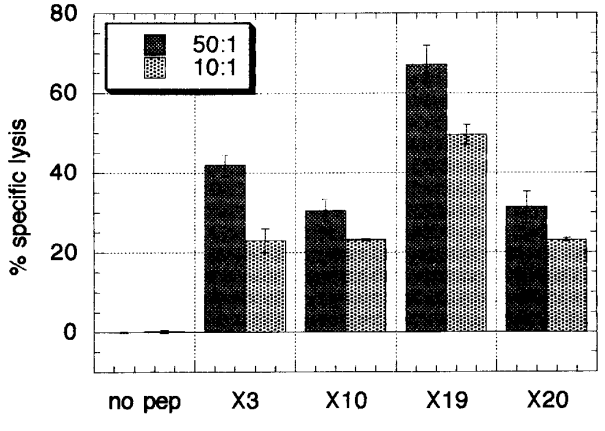

FIG. 3. The frequency of TZ effectors specific for Tax peptides in the limiting dilution analysis (see Fig. 2 and Table 3) is mirrored in the specific lysis of peptide pulsed targets by bulk T-cell lines. T-cell lines were tested at 10–30 days after nonantigen stimulation *in vitro* on peptide-treated autologous B-cell lines. Percentage specific lysis is shown at effector:target ratios of 50:1 and 10:1 for each peptide-treated target. Error bars represent ± standard error of the mean of duplicate values.

frequencies to several peptide epitopes were studied over a period of 5 months. We found that while peptide X19 was always immunodominant, CTL effector frequencies to other peptides fluctuated with time (data not shown) although the range of frequency values were always similar (between 1/250 to 1/4000). This suggests a dynamic process of viral protein expression and consequent response of the CTL effectors, and emphasizes the difficulty in drawing conclusions about the total CTL response to all epitopes at one time point. This is reinforced by data from subject HM, where although in recent bulk cultures of $CD8^+$ cells the response to peptide XN3 was immunodominant (data not shown) this has not always been the case (see Parker *et al.,* 1994). When assayed by limiting dilution, the effector frequency for peptide XN3 in HM was slightly lower than that for peptide XN11.

The cumulative effector frequency for all epitopes measured in TZ was very high (e.g., 1/129, Table 3). This figure should be interpreted with caution, because (1) subject TZ has had an unusually rapid progression of disease, and so lies at one extreme of the HAM/TSP clinical spectrum; (2) we have not measured the total effector frequency of anti-Tax CTL in other subjects; (3) we do not know the dynamic nature of responses in other individuals. We have found that limiting dilution assay using vaccinia recombinant-infected target cells is less sensitive than using peptide-pulsed targets (data not shown), possibly because of competition or antagonism between peptides processed from the recombinant protein.

The CTL frequencies per epitope measured in HTLV-1-infected patients were higher than those determined in other virus infections (Borysiewicz *et al.,* 1988) except for HIV-1, where frequencies of 1/3000 to 1/30,000 peripheral blood lymphocytes specific for HIV-1 p24 antigen were reported (Gotch *et al.,* 1990). The higher effector frequencies in HIV-1 and HTLV-1 are consistent with the presence of a CTL response in freshly isolated T cells in a high proportion of patients and with persistent expression of viral proteins.

Fresh CTL assays measure specific lysis of targets by circulating, activated CTL in the periphery. These are stimulated *in vivo* by newly synthesized antigens in acute and persistent virus infections. Memory CTL, which are quantified by limiting dilution assay, may persist for years after clearance of the virus (Zinkernagel *et al.,* 1993) and can be assayed only after antigen-specific restimulation. Subjects HM, HW, HZ, TD, and TZ all had activated CTL (fresh responses) and high numbers of memory CTL to tax epitopes. Subjects HS and TV had low or no activated CTL (no detectable fresh response) but normal numbers of memory CTL (T-cell line and LDA, $f = 1/3141$ and $f = 1/2618$, respectively). In contrast, subject TY had low or no activated CTL (no detectable fresh response) and few memory CTL (no response detected in bulk T-cell line and LDA $f = 1/5838$). In these subjects, therefore, an active CTL response in fresh blood was associated with a higher effector frequency of Tax-specific memory CTL as measured by LDA (Fig. 3, Table 3). This contrasts with other persistent viral infections where fresh CTL can be detected only in the acute phase of the infection.

A CTL response to Rex has not been reported in HTLV-1-infected patients. Tax and Rex are products of the same 2.1-kb mRNA, although it is not known whether infected cells chronically express Rex as well as Tax. In this study, we were unable to find specific CTL responses to Rex peptides in patients who had strong CTL activity to the Tax protein and in some cases to a peptide derived from Pol. We were unable to test for recognition of endogenously produced Rex protein in these experiments and therefore the presence of natirally processed Rex epitopes cannot be excluded. However, the strategy of using peptide pools has been very successful for identifying Tax epitopes and we would not expect the requirement for Rex peptide presentation to be unusual. These data emphasize the striking difference between CTL recognition of Tax and Rex peptides, rather than the complete absence of CTL epitopes in Rex which is not proven. They are consistent with the possibility that Rex is not available in the cytoplasm for presentation to CTL by MHC class I molecules, perhaps because of sequestration in the nucleus or Rex instability.

The pathogenesis of HAM/TSP remains speculative. There is little evidence of HTLV-1 infection of resident cells in the CNS (Hara *et al.,* 1994; Lehky *et al.,* 1995) and although CTL have been found in the CNS (Elovaara *et al.,* 1993), they were not present in a substantially higher frequency than in the same patient's peripheral blood. This argues against selective migration of anti-HTLV-1 CTL into the CNS (Bangham, 1993). If activated T-cells migrate into the CNS irrespective of antigen specificity (Wekerle *et al.,* 1986), CNS damage could be a result of the Tax-driven secretion, by these lymphocytes, of cytokines which are known to be neurotoxic in high concentrations (Sawada *et al.,* 1992). Similarly, activated T-cells might migrate into other tissues, causing a wide spectrum of HTLV-1-associated inflammatory diseases (Daenke and Bangham, 1994).

In summary, we have found a strong CTL response to Tax but not Rex in the peripheral blood lymphocytes of healthy carriers and HAM/TSP patients. Both groups show similar proportions of subjects with high or low contribution of activated and memory CTL to Tax. As the frequency, antigen specificity and activation state of Tax-specific CTL are the same, these data do not support an association between high CTL activity and the development of HTLV-1 neurological disease.

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