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Circulating Anti-Tax Cytotoxic T Lymphocytes from Human T-Cell Leukemia Virus Type I-Infected People, with and without Tropical Spastic Paraparesis, Recognize Multiple Epitopes Simultaneously

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CD8$^+$ T cells were freshly isolated from a human T-cell leukemia virus type I (HTLV-I)-infected patient with tropical spastic paraparesis. These cells, which were specific for HTLV-I Tax, simultaneously recognized a minimum of five, and possibly as many as seven, distinct peptide epitopes within the protein. A further Tax epitope was recognized after a short period of culture without exogenous peptide stimulation. All but one of these epitopes were clustered in the N-terminal third of Tax, and one of the epitopes was clearly immunodominant on two separate occasions of testing. Recognition of the immunodominant epitope was restricted by human leukocyte antigen (HLA) B15, and recognition of all the others was by HLA A2. Similar patterns of cytotoxic T lymphocyte recognition of the HLA A2-restricted Tax peptides in two healthy HTLV-I-seropositive individuals, each of whom carried the HLA A2 allele, were observed.

Human T-cell leukemia virus type I (HTLV-I) infection causes disease only rarely. Seroepidemiological studies have estimated that 1 to 2% of infected people develop either tropical spastic paraparesis (TSP)/HTLV-I-associated myelopathy (HAM) (12) or adult T-cell leukemia (21). More recently, other inflammatory disorders have been associated with infection: polymyositis (20), chronic infective dermatitis (18), Sjogrens syndrome (31), and occasionally, T-cell alveolitis (28). There are also reports of occasional noninflammatory conditions in HTLV-I-seropositive patients, such as narcolepsy (9) and a dystrophic myopathy (1b), although insufficient data exist to determine whether these are coincidental findings or genuine associations with HTLV-I. It is possible that estimates of the frequency of HTLV-I-associated disease will need to be increased in the light of molecular, rather than just serological, evidence of infection (1a).

A frequent finding in HTLV-I-infected people is the presence in their circulation of already-activated CD8$^+$ T cells (cytotoxic T cells [CTL]) specific for the regulatory protein Tax (9, 24). These are capable of directly killing Tax-expressing autologous (or major histocompatibility complex [MHC] class I-matched) target cells in vitro, without the need for repeated stimulation, but it is not known whether the CD8$^+$ cells actually cause cytolysis in vivo. Although activated CTL initially appeared to be characteristic of neurological disease (9), they have also been found in a healthy carrier and in a patient whose dystrophic myopathy may be coincidental to HTLV-I infection (24). Kannagi et al. (11) have reported a patient with TSP/HAM who did not have activated, circulating CTL but from whose peripheral blood lymphocytes (PBL) Tax-specific CTL lines could be generated by repeated in vitro stimulation with HTLV-I-expression T cells.

Thus, there is not a straightforward correlation between the presence of circulating, activated, Tax-specific CD8$^+$ T cells and disease state, although there is some evidence that the frequency of precursor CTL may be greater, on average, in patients with TSP than in asymptomatic patients (2). This may reflect the higher viral load usually found in patients with TSP, as evidenced by their typically higher antibody titer, both to Tax and to structural proteins (27), and greater frequency of HTLV-I proviral genome (16, 32).

It is not clear why Tax is immunodominant for circulating HTLV-I-specific CTL. One reason could be its chronic expression, independently of other HTLV-I genes, as Tax-Rex mRNA is regularly detected in fresh PBL from infected people (4, 15). An additional possibility is that Tax is readily degraded, as protein degradation has been shown to positively correlate with CTL antigenicity in other viral infections (29).

Constant expression of Tax protein would be expected to elicit and maintain a strong CTL response. As evidence for this, we report the occurrence in three HTLV-I-seropositive individuals, only one of whom has HTLV-I-associated disease, of activated, circulating CTL which simultaneously recognize multiple peptide epitopes in Tax.

MATERIALS AND METHODS

Subjects. Results on three unrelated HTLV-I-seropositive individuals, TE, HH, and HM, are presented. Previous studies of HTLV-I-specific CTL from TE have been reported (24), but HH and HM were recently recruited, and their CTL responses have not been described previously. Nota bene: the prefix T refers to the presence of TSP, and H refers to its absence, and the code bears no relation to the subjects' own initials.

TE. TE was a 49-year-old female Afro-Caribbean neurology patient with a 12-year history of TSP, who had been resident for 20 years in the United Kingdom. In October 1991, following a series of urinary tract infections, her neurological condition deteriorated, and she developed paranoid delusions and auditory hallucinations. She responded to antipsychotic medication, in-patient antibiotic treatment, urinary catheterization,

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and the temporary discontinuation of muscle relaxants, but the consequent return of severe spasticity rendered her virtually immobile. By October 1992, she had developed new spasticity and weakness in her upper limbs, especially the right. In November 1992, anemia of chronic disease was diagnosed, full hematological investigations including bone marrow biopsy having excluded a peripheral leukemia/lymphoma. Her peripheral blood showed relative lymphocytosis, with atypical lymphocytes, characteristic of chronic viral infection. She died unexpectedly in February 1993 of bronchopneumonia. Postmortem revealed bilateral consolidation of the lungs and severe lower limb contractures. Brain and upper spinal cord were formalin fixed for subsequent histological examination. TE was bled on six occasions over 2 years. Human leukocyte antigen (HLA) class I type: A2, 30; B15 (subtype 62), 70; Cw10; Bw6; the HLA typing includes minor revisions from that previously reported (24).

HH. HH was a healthy, 22-year-old female Afro-Caribbean student found to be seropositive during routine screening for blood donation. She was bled for CTL assay on one occasion HLA class 1 type: A2, 1; B7, 35; Bw6; Cw4, 7.

HM. HM was a healthy, 45-year-old white Caucasian female whose HTLV-I seropositivity was noted on routine screening for potential blood donation and whose contact tracing and rising serological titer over 6 months strongly suggested recent infection. She was bled for CTL assay on two occasions, ~3 months apart. HLA class 1 type: A2, 1; B8, 14; Bw6; Cw7.

CTL assays. CTL assays were standard 5-h ^{51}Cr-release assays (24); experimental and total counts were measured from duplicate wells, and background counts were measured in quadruplicate wells. Background counts were less than 25% of total counts in all cases.

(i) Target cells. Target cells for identification of epitopes in CTL assays were autologous Epstein-Barr virus-transformed B-cell lines. For determining the HLA restriction of epitopes, allogeneic B-cell lines matched for various MHC class I alleles were used in addition. Prior to incubation with effector cells, targets were chromium labelled for 1 h, washed three times, preincubated with 50 μM peptide (or medium alone) for 1 h, and then washed again before being used in an assay.

(ii) Effector cells. Effector cells were CD8$^+$ T cells from TE, HH, and HM used immediately after isolation or following in vitro culture. Cultured CTL were washed twice before use in assays, to remove interleukin-2 from the medium.

(iii) Student's *t* tests. Student's *t* tests were used to compare the mean percent specific lysis of test targets with the appropriate negative control. A pooled estimate of the standard deviation, based on the negative control and the particular target being evaluated and their respective degrees of freedom, was used in each case. The significance level for the *t* value obtained was set at 5%, corrected as necessary for the number of comparable targets tested in parallel.

Isolation of fresh CD8$^+$ T cells. Using standard techniques, PBL, containing an estimated 20% CD8$^+$ T cells, were separated from whole, heparinized blood with lymphocyte separation medium (Flow Laboratories). CD8$^+$ T cells were positively selected, as described elsewhere (24), by incubating PBL with M-450 anti-CD8 Dynabeads (Dynal) for 20 min on ice, at a ratio of two beads per CD8$^+$ cell. The beads were retained against a magnet during three washes with RPMI 1640–1% fetal calf serum, and the supernatant, depleted of CD8$^+$ T cells, was subsequently used as a source of autologous, CD4-enriched feeder cells for CD8$^+$ T-cell lines. The CD8$^+$ T cells remaining were stripped off the Dynabeads after a 1-h incubation at room temperature with goat anti-sheep immunoglobulin G (Detachabead), resuspended in RPMI 1640–10% fetal calf serum, and used either (i) directly in a fresh CTL assay or (ii) for setting up CTL lines. Positively selected CD8$^+$ T cells were 98% pure on fluorescence-activated cell sorter analysis (data not shown).

CTL lines. Freshly isolated CD8$^+$ T cells (1×10^6 to 2×10^6) were plated in 2 ml of RPMI 1640–10% fetal calf serum, at a ratio of 1:1 with autologous, irradiated CD4-enriched feeder cells from the same time point. Four days later, they were expanded with phytohemagglutinin (PHA15; Wellcome) at a final concentration of 1 μg/ml, and Lymphocult-T (Biotest Diagnostics) at 10% was added to the medium. The cultures were maintained by two or three feedings weekly with 10% Lymphocult-T, without exogenous Tax peptide stimulation.

Peptides. Overlapping peptides spanning the length of Tax, used for fine mapping of epitopes, were 15-mers overlapping by 5 amino acids, synthesized commercially (CRB, Cheshire, United Kingdom). Peptides XN1 to XN12 spanned the N terminus of Tax, X1 to X13 spanned the middle, and X14 to X23 spanned the C-terminal third.

Shorter peptides within the X4 and XN9 Tax 15-mers, based on their common LXPPIT motif, were also synthesized. X4 group: YLYQLSPPI, YLYQLSPPIT, MYLYQLSPPI, QLSP PITW, YQLSPPITW, LYQLSPPITW, and YLYQLSPPITW. XN9 group: TLKVLTPPI, TLKVLTYPPIT, and KTLKVLT PPI.

RESULTS

Previous work (24), using vaccinia virus recombinants, had mapped CTL epitopes to the middle of Tax but had not excluded their presence in the N terminus. With peptides spanning both the N and the C termini, we exploited the high frequency of circulating anti-Tax CTL to map many of the remaining epitopes in fresh assays, prior to distortion of the natural pattern of CTL specificities by PHA treatment in culture. The decision to maximize information about the pattern of peptide recognition of freshly isolated CTL was also a precaution against (i) loss of specificity or activity that might occur during freeze-thaw procedures and (ii) loss of viability of CD8$^+$ T cells, and hence loss of CTL with unknown specificities, due to premature dilution of CTL concentration, as we had previously observed that dilution of CD8$^+$ T cells from HTLV-I-seropositive individuals early in culture, even in the presence of feeder cells and after PHA expansion, sometimes led to rapid cell decline.

To determine whether the initial finding of simultaneous recognition of multiple Tax epitopes was unique to TE (the patient with TSP), we studied two other HTLV-I-infected people (HH and HM) who did not have TSP but who shared HLA A2 with TE.

CTL from HTLV-I-seropositive individuals with and without TSP simultaneously recognize multiple Tax epitopes. (i) CTL responses of TSP patient (TE). Activated anti-Tax CTL, freshly isolated (October 1992) from the peripheral blood of TE, simultaneously recognized at least four distinct peptide epitopes within the protein, as illustrated in Fig. 1a (i). In addition to the previously identified Tax 12-19 octamer from the N terminus and the X4 epitope in the middle region of Tax (24), both groups of peptides (NG1 and NG2) spanning the remainder of the N terminus of the protein were recognized in the same assay. The response to the NG2 group was predominant.

In order to dissect the responses to these two groups of peptides, we reassayed the CTL 3 days later, prior to their expansion with PHA, and before the addition of exogenous interleukin-2. Figure 1a (ii) shows that two peptides (XN3 and

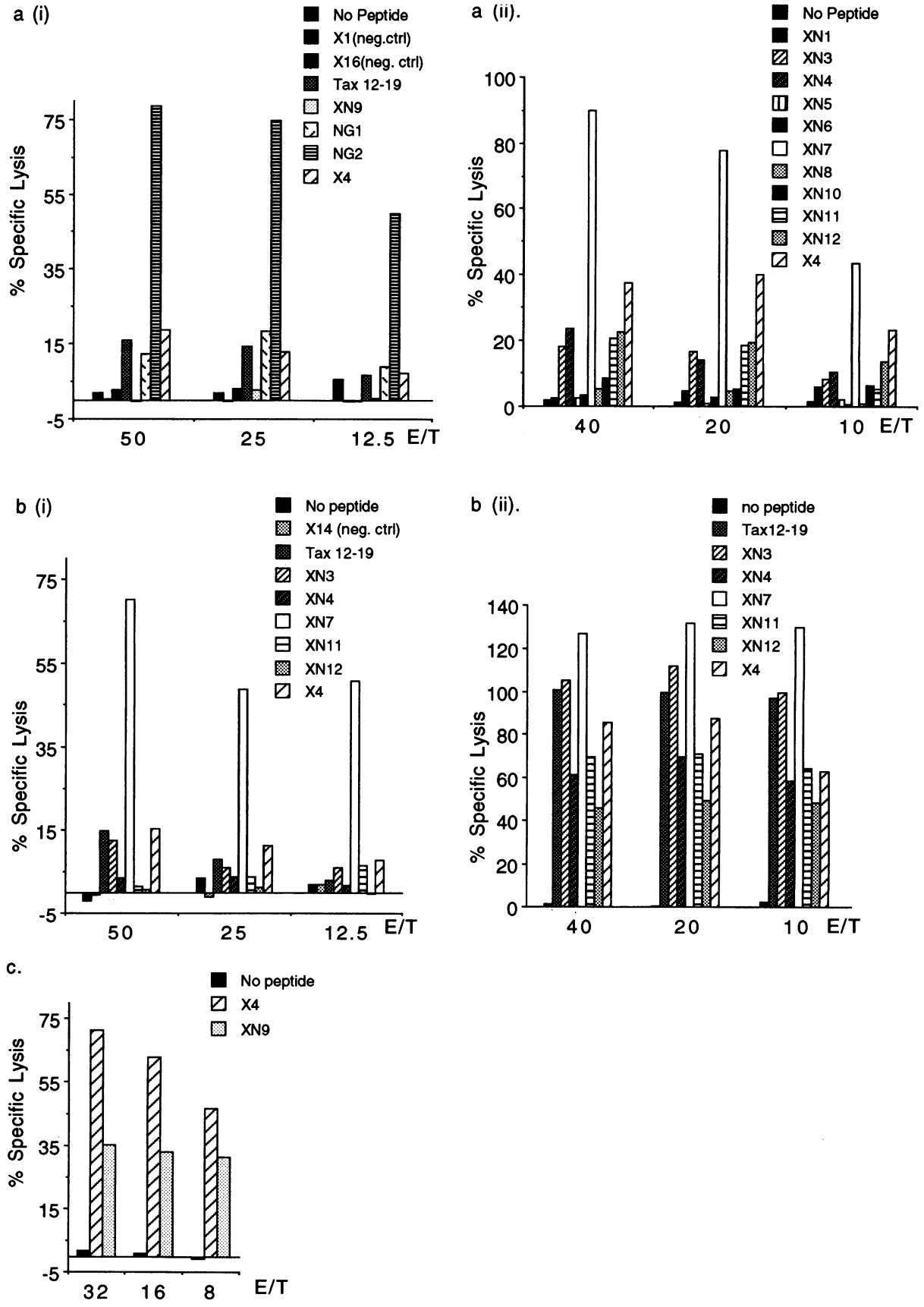
a (i)
No Peptide
X1(neg.ctrl)
X16(neg. ctrl)
Tax 12-19
XN9
NG1
NG2
X4
% Specific Lysis
75
55
35
15
-5
50
25
12.5 E/T
a (ii).
No Peptide
XN1
XN3
XN4
XN5
XN6
XN7
XN8
XN10
XN11
XN12
X4
% Specific Lysis
100
80
60
40
20
0
40
20
10 E/T
b (i)
No peptide
X14 (neg. ctrl)
Tax 12-19
XN3
XN4
XN7
XN11
XN12
X4
% Specific Lysis
75
55
35
15
-5
50
25
12.5 E/T
b (ii).
no peptide
Tax12-19
XN3
XN4
XN7
XN11
XN12
X4
% Specific Lysis
140
120
100
80
60
40
20
0
40
20
10 E/T
c.
No peptide
X4
XN9
% Specific Lysis
75
55
35
15
-5
32
16
8
E/T

TABLE 1. Amino acid coordinates and sequences of the Tax epitopes identified to date in three HTLV-I-seropositive individuals

Epitope	Amino acids	Sequence	HLA restriction	Subject(s)
Tax 12-19	12–19	LFGYPVYV	A2[a]	TE, HH, HM
XN3	21–35	GDCVQGDWCPISGGL	A2	TE, HH, HM
XN4	31–45	ISGGLCSARLHRHAL	A2	TE, HH, HM
XN7	61–75	GRVIGSALQFLIPRL	B15	TE
XN9	80–95	TQRTSKTLKVLTPPIT	A2	TE, HH, HM
XN11	101–115	IPPSFLQAMRKYSPF	A2	TE, HH, HM
XN12	111–125	KYSPFRNGYMEPTLG	A2	TE, HH, HM
X4	151–165	VVCMYLYQLSPPITW	A2[b]	TE, HH, HM
X7	181–195	AFLTNVPYKRIEELL	B14[c]	HM

[a] From reference 30.
[b] From reference 24.
[c] The B14-restricted nonamer, VPYKRIEEL, identified in references 2 and 17.

XN4) within the first group and three (XN7, XN11, and XN12) within the second group were recognized. Peptide XN7, spanning amino acids 61 to 75, was clearly immunodominant and responsible for the immunodominance of the NG2 peptide group on fresh assay. Details of these peptides are shown in Table 1. Other peptides within the original groups (XN1, XN5, XN6, XN8, and XN10) were clearly not recognized. This represented a repertoire of seven distinct Tax epitopes simultaneously recognized by circulating, activated CTL in TE, although it remained a possibility that the overlapping pairs XN3-XN4 and XN11-XN12 each shared a single epitope, reducing the number of epitopes concurrently seen to a minimum of five.

We tested the fresh responses of circulating CTL from TE again (November 1992), 1 month after the testing just described. As illustrated in Fig. 1b (i), the dominance of activated CTL specific for the Tax epitope XN7 was undiminished, but the pattern of recognition of the Tax epitopes which were less dominant in this individual had changed slightly. CTL specific to Tax 12-19, XN3, and X4 remained readily detectable, but those to XN4, XN11, or XN12, did not. Such changes in the pattern of fresh anti-Tax responses over time were presumed to reflect fluctuations in the frequency of peptide-specific CTL in the peripheral circulation. However, after a brief period of expansion in culture, CTL lines derived from this time point, illustrated in Fig. 1b (ii), responded strongly to the full range of Tax epitopes identified at the previous time point [Fig. 1a (ii)], suggesting that CTL specific to XN4, XN11, and XN12 had in fact been present in the freshly isolated PBL assayed in November [Fig. 1b (i)] but were below the level of detection.

Five weeks after PHA expansion in vitro, CTL without exogenous peptide stimulation were also shown (Fig. 1C) to recognize an additional Tax peptide, XN9, which had not been detected in the fresh assay 1 month previously [Fig. 1a (i)]. The recognition of XN9 and its HLA A2 restriction had been demonstrated in X4-stimulated CTL lines from previous PBL samples from this subject (October and November 1991 [data not shown]) and had initially been interpreted as a cross-reactivity. However, the lack of reactivity to short synthetic peptides (Materials and Methods) based on the common LXPPIT motif (Table 1) suggested that this was not a cross-reactivity, bringing the total number of Tax peptides recognized by TE CTL to a minimum of six and a maximum of eight, all but one of which clustered in the N terminus.

The pattern of fresh CTL responses to the overlapping pairs of peptides XN3-XN4 and XN11-XN12 observed at different time points, and subsequently observed in other HTLV-I-infected individuals (see below), when sometimes only one of an overlapping pair was recognized, suggested that each peptide within a pair contained a distinct epitope. Recent preliminary studies using limiting dilution cloning of the CTL responses of subject HM support this (1).

HLA restriction. Early experiments using cultured CTL lines from TE, derived from the time points described above, had shown that all the newly defined epitopes were restricted either by HLA A2 or HLA B15, as illustrated for XN7 in Fig. 2a (i) and XN3 and XN4 in Fig. 2a (ii). Subsequent assays of additional CTL lines from these time points, Fig. 2b (i to iii), showed that recognition of XN7 was restricted by HLA B15 and that of XN3, XN4, XN11, and XN12 was restricted by HLA A2. The HLA restriction of these epitopes was consistent with the CTL responses subsequently observed in HH and HM (below), who shared HLA A2, but not HLA B15, with TE.

(ii) CTL responses of healthy HTLV-I carriers. Freshly isolated CTL from HH and HM were tested against targets infected with the vaccinia virus recombinants described previously (24), expressing whole and partial HTLV-I genes (data not shown), in parallel with targets incubated with the Tax peptides described below. In both subjects, only products of the Tax gene were significantly recognized, but the pattern of responses to the Tax deletion recombinants suggested that the epitope(s) recognized by fresh CTL from HH was concentrated in the N terminus of Tax, whereas those recognized by HM were also located in the middle of the molecule.

HH. Freshly isolated CTL from a single peripheral blood sample of HH (March 1993) simultaneously recognized three (Tax 12-19, XN3, and XN12) of the HLA A2-restricted epitopes previously defined for TSP patient TE, as shown in Fig. 3a (i). Smaller levels of lysis to XN4, XN11, and X4 did not reach the required statistical criterion but may have been real, as suggested by the responses of CTL lines derived from this time point (below).

Assay of PHA-expanded $CD8^+$ T cells (day 21) confirmed specificities to each of the N-terminal epitopes recognized

FIG. 1. Multiple peptides in HTLV-I Tax were recognized simultaneously by already-activated CTL from an HTLV-I-infected subject (TE) with TSP. (a) (i) Fresh $CD8^+$ T cells (October 1992) recognized peptides X4 and Tax 12-19 from the middle and N terminus of Tax, respectively, and both groups of overlapping peptides spanning the remainder of the N terminus: NG1 (XN1, XN3, XN4, XN5, and XN6, but not XN2, which contained the Tax 12-19 sequence) and NG2 (XN7, XN8, XN10, XN11, and XN12, but not XN9, which was tested individually in the same assay). Peptides X1 and X16 had never been recognized by CTL lines from this subject previously and were included as negative controls. (a) (ii) Dissection of the responses to groups NG1 and NG2 at 3 days later, before expansion with PHA, showed that they represented recognition of five further peptides, of which XN7 was clearly immunodominant (XN3, XN4, XN11, and XN12, significant at $P = 0.05$, and XN7 significant at $P < 0.005$). Peptide X4 was included as a positive control. (b) (i) One month later (November 1992), fresh CTL simultaneously recognized four Tax epitopes described above (Tax 12-19, XN3, XN7, and X4); XN7 remained immunodominant, but specificities to XN4, XN11, and XN12 were not detected. Peptide X14 was the negative control. (b) (ii) On day 14 of culture, after PHA expansion, $CD8^+$ T-cell lines derived from the November sample showed strong responses specific to all the peptides recognized 1 month previously, including XN4, XN11, and XN12. (c) On day 35 of culture, CTL specific for an additional peptide (XN9) in the N terminus of Tax were detected, without exogenous peptide stimulation.

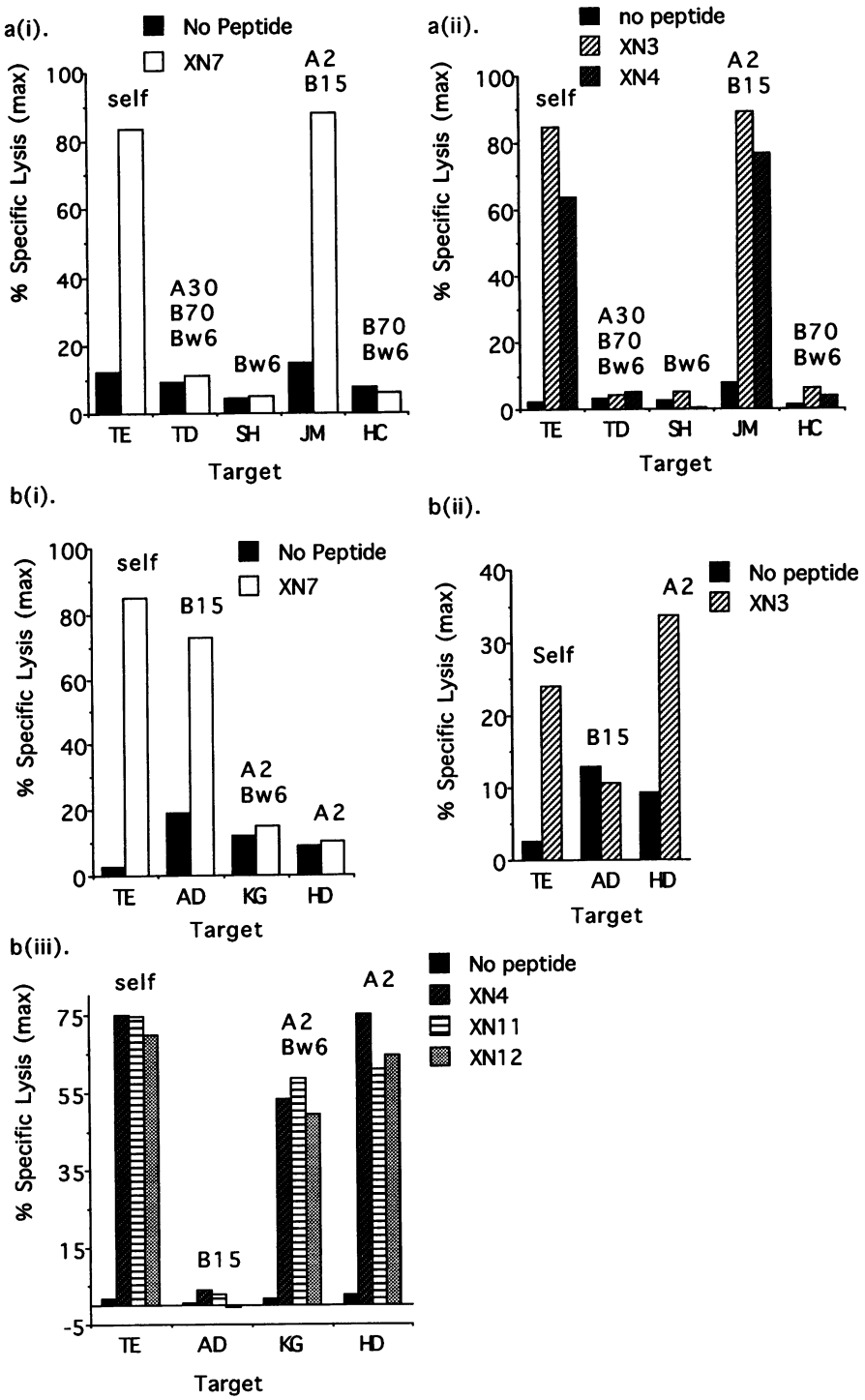

FIG. 2. HLA restriction of newly identified Tax epitopes in TE. Tax epitopes XN3, XN4, XN11, XN12, and XN7 were all shown to be restricted by HLA A2 or HLA B15, as illustrated in panel a (i) for XN7 and panel a (ii) for XN3 and XN4. CTL assays in b (i) to b (iii) showed that XN7 was restricted by HLA B15 and that XN3, XN4, XN11, and XN12 were each restricted by HLA A2. The class 1 alleles matched against TE are indicated above the appropriate histograms. Full HLA class 1 types of target B-cell lines are as follows: TD[A29/30, B17/70, Cw2(6), Bw4/6]; SH[A1/31, B8/27, Bw4/6]; JM[A2, B15/51]; HC[A23/28, B7/70, C3/7, Bw6]; AD[A9/19, B12/B15]; KG[A2/24, B18/44, Cw3, Bw4/6]; and HD[A2/25, B38/57, Bw4, Cw1/7].

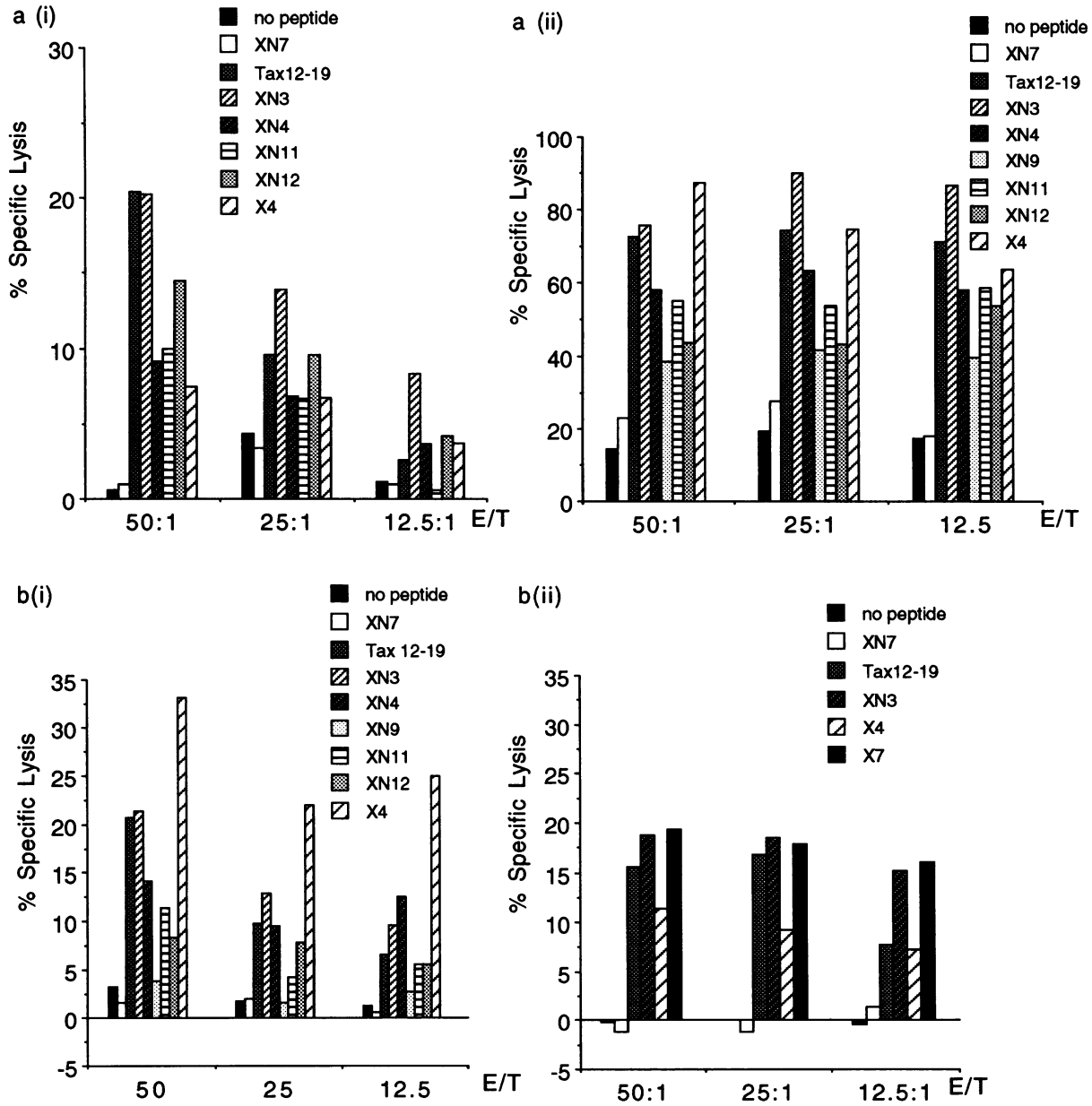

FIG. 3. Two healthy HTLV-I-infected subjects, HH and HM, also recognize multiple peptide epitopes in Tax. (a) (i) Freshly isolated $CD8^+$ T cells from HH, who carried HLA A2, simultaneously recognized three peptide epitopes from the N terminus of Tax (Tax 12-19, XN3, and XN12). Peptide XN7, restricted by HLA B15, was included as a negative control in panels a and b, as neither subject carried this allele. (a) (ii) Three weeks after PHA expansion of HH CTL lines in vitro, these specificities were confirmed; in addition, CTL responses specific for other peptides in the N terminus (XN4, XN11, and XN9), as well as a strong response to the X4 epitope in the middle of Tax, had emerged. (b) (i) Freshly isolated $CD8^+$ T cells from HM, who also carried HLA A2, recognized four peptide epitopes from the N terminus (Tax 12-19, XN3, XN4, and XN11), as well as X4 from the middle third of the molecule. (b) (ii) Approximately three months later, freshly isolated CTL from HM still recognized Tax 12-19, XN3, and X4 but were also shown to recognize an additional peptide, X7, from the middle of Tax. As X7 contained a known HLA B14-restricted nonamer (2, 17), its recognition was consistent with the carriage of HLA B14 by HM.

freshly, as shown in Fig. 3a (ii). In addition, CTL specific to other peptide epitopes in the N terminus (XN4, XN9, and XN11) and to X4 in the middle of Tax had emerged, responses which had been below the level of detection on fresh isolation.

HM. As shown in Fig. 3b (i), fresh $CD8^+$ T cells from the first sampling of HM (March 1993) simultaneously recognized four (Tax 12-19, XN3, XN4, and XN11) of the cluster of epitopes in the N terminus previously defined for TE, as well as the X4 epitope from the middle of Tax. At this time point, the X4-specific response appeared predominant in this subject. A low level of lysis to XN12 did not reach statistical significance, and XN9 and XN7 were not recognized.

Reassaying the CTL 4 days after isolation, prior to PHA and interleukin-2 treatment, against the same selected peptides and additional groups spanning the remainder of Tax, confirmed the findings of the fresh assay but also indicated the

presence of an additional epitope(s) in the middle of Tax (data not shown). The additional peptide group recognized included X7, a Tax 15-mer containing a known HLA B14-restricted nonamer (2). As HM carried HLA B14, recognition of X7 was directly tested in a subsequent fresh assay ~3 months later (May 1993), in parallel with the previously identified epitopes. Both X7 and X4 from the middle of Tax were simultaneously recognized, along with Tax 12-19 and XN3 from the N terminus, as shown in Fig. 3b (ii). Although the overall levels of lysis were somewhat lower than those in the previous fresh assay, each peptide response was statistically significant.

Distribution of peptide epitopes in HTLV-I Tax. Table 1 summarizes the amino acid coordinates and sequences of Tax epitopes demonstrated in the subjects above. All the epitopes shown are clustered in the N terminus and the middle of the molecule, and no peptide epitopes in the C-terminal third of Tax were confirmed for any of these subjects.

DISCUSSION

The anti-Tax specificity of circulating $CD8^+$ T cells from a group of HTLV-I-infected individuals has already been documented (24). Here, we present the results of extended studies on a TSP subject, TE, and in addition, the CTL specificities of two healthy HTLV-I carriers, HH and HM, not previously studied. The main findings were that (i) multiple peptide epitopes in Tax were simultaneously recognized by activated, circulating CTL in subjects with and without TSP; (ii) the HTLV-I-infected subjects presented above had the HLA A2 class 1 allele in common, and the patterns of anti-Tax CTL responses observed reflected class 1 type, rather than the presence or absence of TSP, suggesting that there was not a simple qualitative relationship between the fine specificity of activated CTL and established neurological disease; (iii) although HLA A2 could present several Tax epitopes in an infected person, other class 1 molecules could present Tax simultaneously within the same individual; and (iv) the Tax epitopes recognized within each individual showed marked clustering toward the N terminus and, to a lesser extent, the middle of the molecule.

Multiple CTL epitopes in the same viral protein. The simultaneous recognition of multiple Tax epitopes by fresh $CD8^+$ T cells from HTLV-I-infected individuals implies a high frequency of already-activated, peptide-specific CTL in their circulation, evidence of persistent Tax expression. It is likely that the epitopes described above underestimate the full repertoire recognized, as the peptides available for mapping, 15-mers overlapping by five amino acids, do not include all possible epitope sequences. The decision to use freshly isolated CTL and CTL lines maintained at high cell density may have facilitated the detection of multiple peptide specificities. Recognition of multiple epitopes within the same viral protein, which characterized the activated anti-Tax CTL described above, is not commonly found in other chronic viral infections. This may partly reflect the typically lower circulating frequency of antiviral CTL in other infections (with the exception of human immunodeficiency virus [HIV]) and the need to stimulate and repeatedly restimulate antiviral CTL in vitro to detect their antigen specificity. Such in vitro protocols may select predominant CTL specificities.

We had previously shown that circulating CTL from the HTLV-I-infected subject TE simultaneously recognized at least two Tax peptides restricted by HLA A2: Tax 12-19 in the N terminus (first described elsewhere [30]) and X4 in the middle of the molecule (24). The data presented above extend the number of Tax epitopes recognized simultaneously by this individual to a minimum of six and probably as many as eight. Recognition of more than one epitope within a viral protein has also been described for anti-Gag CTL from primary, unstimulated PBL of HIV-seropositive individuals (10) and for primary anti-Nef CTL extracted from lymphoid foci of active HIV replication (6). It has also been documented for hepatitis B virus nucleocapsid-specific CTL lines, derived from the PBL of a patient with acute hepatitis B infection (19), and for HLA A11-restricted EBNA-4-specific CTL from some individuals infected with Epstein-Barr virus (3). The demonstration of primary, unstimulated CTL responses to a range of epitopes within a single viral protein, such as HIV Nef and Gag or HTLV-I Tax, suggests that persistent expression of viral antigen is responsible for the multiple peptide specificities identified.

HTLV-I Tax also shares with HIV antigens such as Gag, Env, and Nef (23) the capacity to be presented by a variety of class 1 molecules and to generate multiple peptides restricted by the same class 1 allele. This may contribute to the immunodominance of Tax for CTL in HTLV-I infection. Even in a single individual, Tax can be presented by more than one class 1 allele simultaneously, as shown by the HLA B15-restricted XN7 epitope described above for TE, which is situated within the HLA A2-restricted cluster in the N terminus of Tax. Recently, Koenig et al. (17) have shown that in different individuals with HTLV-I-associated neurological disease, Tax can be presented by HLA A3, HLA A2, or HLA B14, depending on the patient's class 1 type. However, the simultaneous presentation of Tax by different class 1 antigens in a single individual and the multiple recognition of different Tax epitopes restricted by the same class 1 antigen were not observed. The restricted range of CTL epitopes identified by Koenig et al. within a given HTLV-I-infected individual may have reflected the in vitro protocols chosen, as discussed above.

Multiple CTL epitopes in Tax and immune surveillance. In HTLV-I, the predominantly anti-Tax specificity of circulating CTL may be important in limiting the extent of active viral replication, because continual availability of activated CTL, specific to a regulatory protein expressed early in cellular infection, may abort otherwise productive cycles of HTLV-I replication. Such is the case in simian virus 40 (13) and adenovirus E1 infections (14), in which CTL responses to viral oncogenes (the large T antigen and E1A protein, respectively), expressed early in the infective cycle, are associated with resistance to the spread of malignantly transformed cells. In Epstein-Barr virus infection, virus-specific CTL surveillance directed against different latency-associated antigens (22) and in some individuals against multiple epitopes within the same antigen (3) controls persistent infection of B lymphocytes and is thought to limit the development of Epstein-Barr virus-associated malignancies. In HTLV-I infection, a wide repertoire of anti-Tax specificities at the peptide level would increase the probability that an infected cell in any site would interact with cognate CTL early in the replicative cycle of the virus. This would be particularly important if there were tissue-specific differences in the efficiency of presentation of Tax epitopes.

In addition to the strong anti-Tax responses directed against multiple epitopes observed in fresh CTL from the two healthy carriers, HH and HM, in this study, fresh CTL from an additional healthy carrier (without HLA A2) have also been shown to respond briskly to Tax-expressing targets, although specificity has not yet been mapped at the peptide level (23a). Together with other data (24), this suggests that activated anti-Tax CTL are a frequent finding in healthy HTLV-I-

seropositive individuals. In these studies, the healthy carriers were on average younger than subjects with TSP and may have been infected more recently (as is known to be the case with HM). As TSP develops only rarely in seropositive patients, it raises the possibility that circulating anti-Tax CTL early in the natural history of infection protect against infection of the central nervous system with HTLV-I, contributing to the rarity of disease development. Such has been shown to occur in mice infected with the CasBrM mutant of murine leukemia virus, with virus-specific CTL responses during a critical period early after infection protecting against subsequent neurological disease (7).

It remains unclear whether the predominant role of antiviral CTL in TSP is protective or pathogenetic. It has been suggested that a higher precursor frequency of antiviral CTL in patients with HTLV-I-associated neurological disease compared with that in asymptomatic carriers (2) is consistent with a causal role for CTL in the characteristic central nervous system lesions of TSP. If subsequent studies do support a pathogenetic role for anti-Tax CTL, the design of any peptide-mediated immunotherapy would be highly complex because of the multiplicity of epitopes simultaneously recognized in a given individual. However, the rapid development of TSP in immunosuppressed individuals infected with a high viral load (5, 26) would suggest rather that anti-HTLV-I CTL control the rate and extent of central nervous system involvement. More recent histopathological evidence that $CD4^+$ $CD45RO^+$ T cells, the main reservoir of HTLV-I infection (25), are prevalent in the early lesions of TSP (8), with $CD8^+$ T cells predominating later, would be consistent with a protective role of CTL.

In summary, we have shown that circulating, activated anti-Tax CTL from two healthy HTLV-I seropositive individuals and a seropositive patient with TSP recognized multiple peptide epitopes simultaneously. The qualitative pattern of Tax peptides recognized reflected HLA class 1 type, rather than the presence or absence of TSP, and showed marked spatial clustering within the N-terminal third, and to a lesser extent the middle, of the Tax molecule. Although HLA A2 presented several Tax epitopes simultaneously in each subject, in two of them, other class 1 antigens could also present Tax to CTL, and HLA A2 did not necessarily present the immunodominant epitope within a cluster. Further studies are needed to determine whether the predominant role of anti-Tax CTL in HTLV-I infection is protective or pathogenetic.

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