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Naturally Occurring Variants of Human T-Cell Leukemia Virus Type I Tax Protein Impair Its Recognition by Cytotoxic T Lymphocytes and the Transactivation Function of Tax

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There is a high degree of intraisolate sequence heterogeneity in the *tax* gene of human T-cell leukemia virus type I (HTLV-I), although the sequence variation between patients is small compared with that of human immunodeficiency virus type 1. In the present study, we investigated whether naturally occurring amino acid substitutions changed the properties of the Tax protein in two respects: first, recognition of the protein by cytotoxic T lymphocytes (CTL), and second, the ability of the Tax protein to transactivate various promoters. We found that (i) all of the observed amino acid substitutions that occur in known CTL epitopes abolished the recognition of the synthetic peptide representing the respective epitope; (ii) these substitutions occurred significantly more frequently in subjects carrying HLA-A2; and (iii) most of the amino acid substitutions severely reduced the ability of Tax protein to transactivate three promoters: the HTLV-I long terminal repeat, the c-*fos* promoter, and the interleukin-2 receptor alpha chain promoter.

Cytotoxic T lymphocytes (CTL) are an important part of the immune response against viral infections. Infected cells present a complex consisting of a major histocompatibility complex class I (MHC I) molecule and a viral peptide at the cell surface, which is recognized by the T-cell receptor of $CD8^+$ CTL (for review, see reference 37). This recognition is very specific, and it is well known that CTL can distinguish between different viral strains (6, 18, 39). Most if not all RNA viruses have a quasispecies structure, i.e., consist of a mixture of closely related but not identical viral genomes (13). It has been suggested that CTL escape mutants might arise during viral infection and contribute to the persistence of virus. There is evidence that certain RNA viruses produce variants that escape CTL recognition, both in experimental models (1, 30) and in human immunodeficiency virus (HIV)-infected individuals (29). Two factors seem to favor the emergence of CTL escape mutants: a strong CTL response and a highly variable virus.

The human T-cell leukemia virus type I (HTLV-I) persists for many years in infected individuals, but unlike with HIV, only a minority of them develop disease (adult T-cell leukemia [ATL] [12, 31] or tropical spastic paraparesis [TSP] [2, 11, 25]). Although in HTLV-I infection, sequences differ little between in vivo isolates, intrastrain variability has been shown to be considerable (3, 7, 9, 24). There is also evidence for ongoing virus replication in vivo: Tax protein can be activated by cellular transactivation factors (4, 21), and constant transcription of *tax* mRNA can be detected by PCR and in situ hybridization in healthy seropositive subjects and ATL and TSP patients (10, 15, 23). In addition, activated HTLV-I-specific CTL have been found in the peripheral blood of healthy seropositive subjects and TSP patients (14, 27, 28). These CTL recognize (without in vitro stimulation) the Tax protein and occasionally envelope and polymerase proteins, which indicates that these proteins are chronically expressed in infected individuals. Among patients with the HLA-A2 haplotype, the simultaneous recognition of at least five epitopes on the Tax protein has been demonstrated (28). In this study, we examined naturally occurring mutants of Tax to answer three questions. (i) Do mutations arise that interfere with CTL recognition of the Tax protein? (ii) If so, do these putative CTL escape mutations in HLA-A2-restricted CTL epitopes arise more commonly in individuals with HLA-A2? (iii) Is there a cost to the virus of these putative escape mutations?

Subjects and methods. The HLA-A2-positive subjects TE, HD, and HM and the non-HLA-A2 subjects have been described elsewhere (24, 27, 28). The first letter of the two-letter patient code indicates the clinical state of the subject: TD, TE, and TG have TSP, while HD, HN, and HM are healthy carriers of HTLV-I.

The preparation of genomic and cDNA was done by standard procedures and has been described elsewhere, as have the primers and conditions for PCR amplification (24).

The CTL assays, T-cell culture, and peptide synthesis were done as described before (26–28). To be recognized as specific lysis, chromium release values had to be 3 standard deviations above the respective control value. To try and grow CTL specific to variant peptide sequences, 2 million cells from an autologous Epstein-Barr virus (EBV)-transformed B-cell line were treated for 1 h with a 50 μM concentration of the respective peptides, irradiated, washed, and added to 1 million $CD8^+$ cells. The line was maintained in culture as described before (27, 28) for 3 weeks before CTL activity was assayed.

Three plasmids were used to test the transactivation activity of the Tax protein: (i) U3R-CAT (35), containing the promoter/enhancer (U3) region of the HTLV-I long terminal repeat (LTR); (ii) −317 IL-2RQ-CAT (33), which contains the promoter of the human interleukin-2 receptor (IL-2R) alpha chain gene; and (iii) pF711CAT (38), which contains the c-*fos* gene promoter.

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TABLE 1. Tax variants[a]

Peptide and variant	Amino acid residues	Peptide sequence	Nucleotide no. mutated	Nucleotide change	No. of times found	Tax clone (CAT assay)
Tax	12–19	LFGYPVYV				
TV1		P-------	7354	T→C	2	C1
TV2		--R-----	7359	G→A	6	C2
TV3		---C----	7363	A→G	3	C3
XN3	21–35	GDCVQGDWCPISGGL				
XN3/V1		--Y----------	7387	G→A	2	C4
XN3/V2		---------S-----	7407	C→T	2	C5
XN3/V3		----------V----	7410	A→G	2	C6
XN4	31–45	ISGGLCSARLHRHAL				
XN4/V1		V--------------	7410			C6
XN11	101–115	IPPSFLQAMRKYSPF				
XN11/V1		--L----------	7627	C→T	2	C7
XN12	111–125	KYSPFRNGYMEPTLG				
XN12/V1		------S--------	7669	A→G	3	C8
XN12/V2		--------C------	7675	A→G	2	C9

[a] Nine distinct nucleotide changes were found to change the amino acid code in five epitopes. The sequences of consensus peptides and variant peptides are given. Nucleotides are numbered according to the numbering in the EMBL database. The frequency is the number of clones carrying a certain nucleotide change (out of the 179 clones sequenced). C1 through C9 are *tax* clones used for the CAT assay, carrying only the respective nucleotide change. The nucleotide change A to G at position 7410 affects the two overlapping epitopes XN3 and XN4. The nomenclature of TV1 to TV3 differs from that used previously (30). XN4 and XN12 were not recognized by CTL from HD.

Either the *tax* variants were derived from fully sequenced, naturally occurring clones, or point mutations were introduced into a clone representing the consensus sequence of *tax* in subject TE by using the T7-Gen in vitro mutagenesis kit (United States Biochemicals). The *tax* variants were cut out of M13mp18 by using *Eco*RI and *Xba*I and ligated into the expression vector pJFE14 (36). A microversion of the chloramphenicol acetyltransferase (CAT) assay relying on the diffusion of reaction products into the scintillation fluid was used as described before (22). For every plasmid, at least two different DNA preparations were tested, and the assay was done at least three times.

The *tax* gene (1,059 bp) was amplified by PCR from one healthy seropositive subject (HM), one seropositive subject with muscular dystrophy (HD), and one TSP patient (TE) who had chronically activated, HLA-A2-restricted Tax-specific CTL responses at all time points tested. For comparison, the genes from three seropositive subjects who did not carry the HLA-A2 allele were also sequenced. Twenty clones were sequenced per time point. Genomic DNA was investigated from all three (HD, HM, and TE) and cDNA from two patients (HM and TE). In addition, TE was followed up over time, and genomic DNA derived from samples taken 5 and 10 months after the first time point was studied. The epitopes presented by HLA-A2 all lie in the amino-terminal half of the Tax protein, and sometimes only the 5′ two-thirds of the *tax* clones were sequenced. As PCR is error prone, only changes which were found in two independent amplifications were considered to be true in vivo mutations.

Sequence variation in CTL epitope-coding regions in HTLV-I Tax. Although the respective consensus *tax* gene sequences of the isolates from the three HLA-A2 and the three non-HLA-A2 subjects differed slightly (at the most in six amino acid residues), within the known CTL epitope-coding

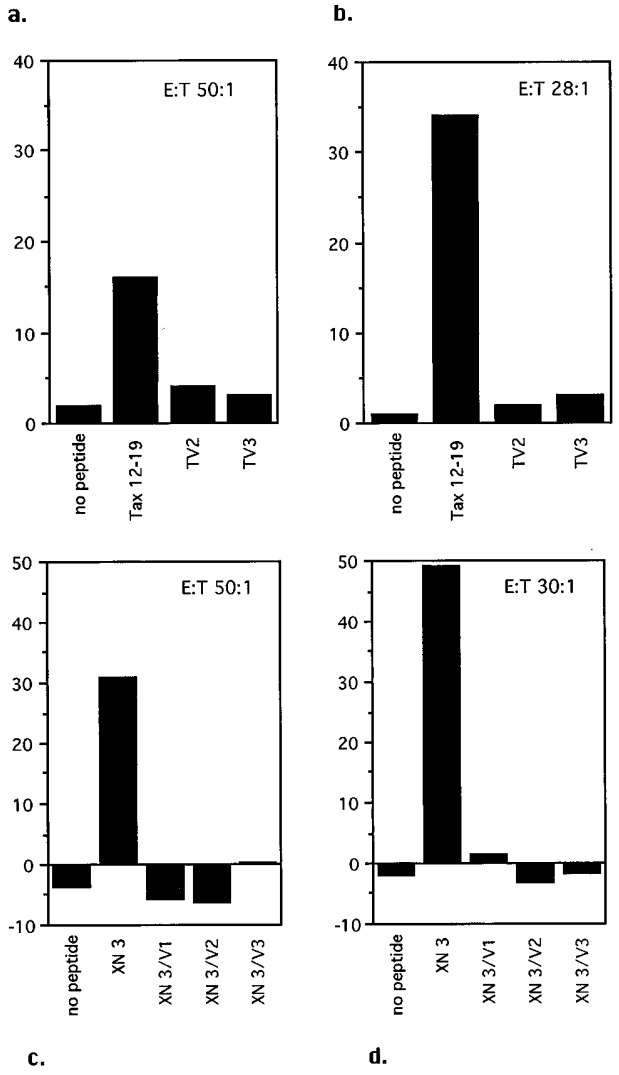

FIG. 1. Variant peptides are not recognized by CTL. The Tax 12-19 epitope and two variants were tested with CTL from TE (top), and the XN3 peptide and its variants were tested with CTL from HM (bottom). Chromium release assays were done with freshly separated CTL (left) or after 19 days in culture (right) at the indicated effector-to-target cell (E:T) ratios.

regions the sequences were identical (Table 1). However, within each isolate, there was considerable sequence heterogeneity among the clones. For non-HLA-A2 subjects, among 116 *tax* clones from genomic and cDNA, only 1 clone had one mutation in a CTL epitope-coding region, whereas in a total of 179 *tax* clones from genomic and cDNA from HLA-A2-positive subjects, 24 clones (13%) had one or more mutations in a CTL epitope-coding region. The difference is highly significant (chi-squared test, $P < 0.001$).

We found nine distinct mutations in five epitopes (Table 1); each individual mutation was found between two and six times. Eight clones had two mutations, and 16 clones had one mutation only. All mutations were found in genomic DNA. The variants C2 and C3 were also found in cDNA, indicating the current transcription of these variants. The most common variant, C2 (six times), was also found in a single clone in a TSP patient without HLA-A2. The number and distribution of coding changes within the epitopes did not obviously differ from neighboring parts on the *tax* gene (data not shown).

Mutations in epitopes are not recognized by CTL. We tested the ability of freshly separated $CD8^+$ T cells from the HLA-A2 patients to recognize the Tax variants without in vitro stimulation. Peptides corresponding to the variant sequences were compared with peptides corresponding to the consensus sequence in a CTL assay. Subsequently, the remaining cells of the sample were cultured for 3 weeks to increase the proportion of Tax-specific CTL. In Fig. 1, typical results for two peptides (tax 12-19 and XN3) and their variants are shown. Freshly separated CTL recognized the tax 12-19 and XN3 peptides, but not the variants (Fig. 1a and c), and although culture increased the lytic activity of CTL, no recognition of variant peptides was demonstrated (Fig. 1b and d). Similarly, all other variant peptides failed to be recognized by CTL specific for the consensus peptides (Table 2).

The question arises whether a CTL response subsequently develops in vivo to the variant Tax sequences observed. We tested the recognition of TV2 and TV3 by CTL from TE more than a year after the variant sequences were first documented. The variant peptides were not recognized, and in vitro stimulation with TV2 and TV3 did not lead to variant-specific CTL.

CTL escape mutations lead to abolished or diminished Tax function. The Tax protein is able to transactivate, besides the LTR of HTLV-I, promoters regulated by NF-κB and various other cellular promoters (for review, see reference 19). The resulting activation of the infected cell is thought to be beneficial for virus replication. We cloned the nine *tax* variants into the expression vector pJFE14 and tested their ability to transactivate the LTR of HTLV-I, the promoter of the IL-2R alpha chain, and the c-*fos* promoter in a CAT assay. As the naturally occurring *tax* clones carrying C1, C4, and C9 also contained other mutations, these mutations were introduced singly into a clone representing the consensus sequence of TE by in vitro mutagenesis. Lysates from cells transfected with the various mutant plasmids were blotted, and the amount of protein expressed was measured by densitometry. All mutant plasmids produced amounts of protein comparable to those of wild-type

TABLE 2. Tax-specific CTL do not recognize variant peptides[a]

Peptide	% Lysis at indicated E:T ratio			
	Fresh CTL		Cultured CTL	
	50:1	10:1	30:1	6:1
None	−3.7	−2.7	−2.1	−3.1
XN4				
wt	22.4	7	50	29.6
V1	−0.8	0.6	10.2	3.3
XN11				
wt	17.6	8.4	34.1	20.5
V1	−2.0	0.6	−3.1	−4.6
XN12				
wt	18.3	6.9	39.4	22.2
V1	−0.7	0.1	2.9	1.2
V2	−2.2	−0.3	0.5	−3.6
None	3.3	1.8	ND[b]	ND
Tax 12-19	20.7	9.8	ND	ND
TV1	0.1	2.2	ND	ND

[a] The wild-type (wt) peptides Tax 12-19, XN4, XN11, and XN12 and their variants were tested in a chromium release assay with CTL from HM, and the resulting lysis is given as a percentage. Assays were done with freshly separated CTL (left) or after 19 days in culture (right). The second effector-to-target cell (E:T) ratio for Tax 12-19 and the TV1 variant was 25:1. Similar results were obtained with cells from HD and TE.

[b] ND, not done.

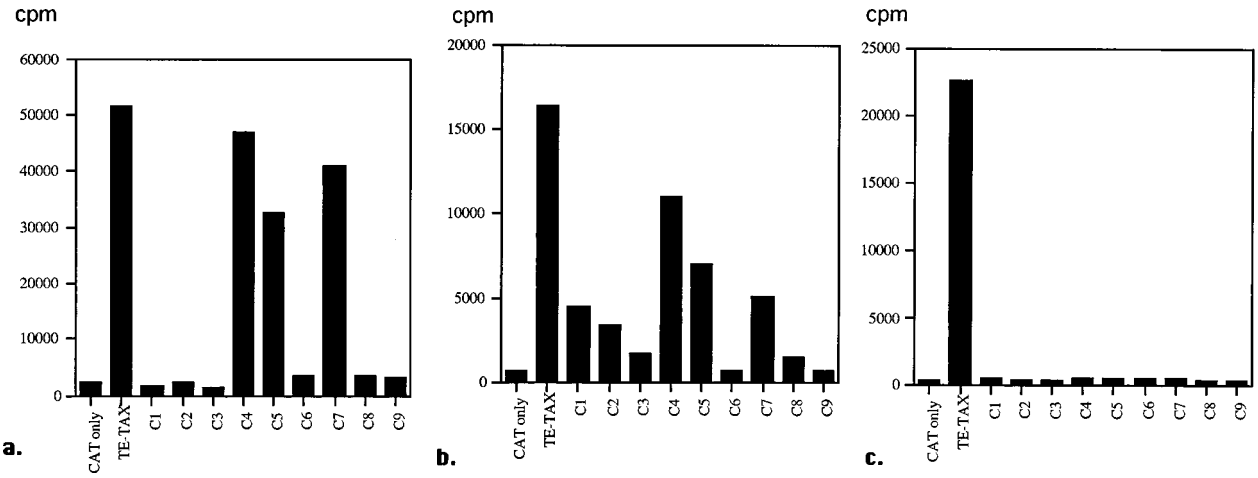

FIG. 2. Variant Tax proteins have reduced or abolished transactivational activity. The *tax* variants tested in CAT assays were naturally occurring clones from subjects HM (C8), HD (C5 and C7), and TE (C2, C3, and C6). Because the changes at nucleotide positions 7354, 7387, and 7675 were on clones with multiple mutations, C1, C4, and C9 were generated by in vitro mutagenesis of the consensus *tax* from TE. All variants and the consensus *tax* were cotransfected with a CAT construct under the control of the LTR (a), the promoter of c-*fos* (b), and the IL-2R alpha chain (c).

Tax except C8, which expressed three times less (data not shown).

Three mutants (C4, C5, and C7) were able to transactivate the LTR but were not as efficient as the consensus Tax (Fig. 2a). The transactivation efficiency for the other mutants (C1, C2, C3, C6, C8, and C9) was at or close to background levels. Similarly, the CAT construct under the control of the c-*fos* promoter was transactivated not at all (C3, C6, C8, and C9) or at levels strongly reduced compared with wild-type Tax (C1, C2, C4, C5, and C7) (Fig. 2b). None of the Tax mutants was able to transactivate the IL-2R alpha chain promoter (Fig. 2c), which is transactivated by interaction of Tax and NF-κB. Comparing the overall efficiency of transcription of the *tax* variants on all three promoters, none was as good as the consensus *tax.*

Two factors probably select against the rise of CTL escape mutants in HTLV-I; one is the simultaneous recognition of multiple epitopes on the Tax protein by CTL (28), and the other is the functional constraints on Tax (32, 34). Most of the *tax* variants seemed to be functionally impaired, but they occurred often enough to be detected within 20 clones. It seems that they have been selected for by the strong CTL pressure on the Tax protein, as they were found almost exclusively in HLA-A2-positive individuals.

Functional impairment of Tax may balance any selective advantage conferred by an amino acid change that abolishes CTL recognition. The naturally occurring Tax variants described here, which are not recognized by CTL, seem to have a severely reduced functional activity. In vitro mutagenesis has shown that the *tax* gene is under strong functional constraints (32, 34): most of the single or double mutations introduced into the *tax* gene impair or abolish *tax* function. When the HIV transactivator (Tat) quasispecies was investigated, 10 to 15% of the *tat* clones turned out to be functionally inactive (20). However, these variants of Tat may still form part of the functional gene pool of HIV, as defective HIV genomes can be rescued by a functional genome after cotransfection (5, 17). The same might be true of HTLV-I infection. Another possible mechanism for maintaining mutations within the epitopes is specific antagonism of CTL recognition of the mutant proteins. Certain variant peptide epitope sequences, which themselves are not recognized by T cells, have been shown to inhibit the recognition of the respective wild-type peptide in vitro. This phenomenon of "peptide antagonism" has been described for both MHC class I- and II-restricted T cells (reviewed in reference 8) and has recently been shown to occur in HIV-1 infection (16).

We wanted to evaluate whether the persistence of HTLV-I infection could be explained by the rise of viral mutants which avoid the CTL response. Our data suggest that mutations do indeed arise as a result of CTL selection, but that the functional cost to the virus of these mutations is very high, as they severely impair the ability of the Tax protein to transactivate different promoters.

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