Detection of Antibodies to trans-Activator Protein (p40\textsuperscript{tax}) of Human T-Cell Lymphotropic Virus Type I by a Synthetic Peptide-Based Assay

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Antibodies to human T-cell lymphotropic virus type I (HTLV-I) trans-activator protein (p40\textsuperscript{tax}) were determined in serum specimens from individuals infected with HTLV-I (n = 138) and HTLV-II (n = 19). Western blot (immunoblot) analysis using recombinant tax demonstrated the presence of anti-tax antibodies in 96% of patients (25 of 26) with HTLV-I-associated myelopathy, 43% of those (20 of 46) with adult T-cell leukemia, and 61% of asymptomatic HTLV-I blood donors (40 of 66); only one of the HTLV-II specimens reacted with the recombinant tax protein. Synthetic peptides (Tax\textsuperscript{806-125}, Tax\textsuperscript{214-233-335}, Tax\textsuperscript{224-331-350} and Tax\textsuperscript{24333-353}) representing the immunodominant epitopes of p40\textsuperscript{tax} detected anti-tax antibodies in 66 (48%), 50 (36%), 66 (48%), and 64 (46%) of 138 HTLV-I-positive specimens, respectively. An enzyme immunoassay using an equimolar ratio of these four peptides allowed sensitive detection of anti-tax antibodies in 96% of patients (25 of 26) with HTLV-I-associated myelopathy, 52% of adult T-cell leukemia patients (24 of 46), and 62% of asymptomatic HTLV-I-infected donors (41 of 66). The synthetic peptide-based cocktail assay was HTLV-I specific, since none of the HTLV-II-infected specimens reacted with these peptides. Interestingly, the corresponding regions from the HTLV-II tax protein, Tax\textsuperscript{806-125}, and Tax\textsuperscript{224-331-350} did not react with either HTLV-II or HTLV-I specimens. Thus, a synthetic peptide-based assay composed of immunodominant epitopes located towards the amino terminus and at the C terminus of p40\textsuperscript{tax} provides a reliable and sensitive assay for the detection of anti-tax antibodies in seroepidemiologic studies.

Human T-lymphotropic virus types I and II (HTLV-I and II) are human retroviruses that have similar genetic structures and modes of action (26). HTLV-I has been shown to be the etiologic agent for adult T-cell leukemia (ATL) and HTLV-I-associated myelopathy (HAM), also known as tropical spastic paraparesis (2, 26); however, most infected individuals remain asymptomatic during the course of their infection. HTLV-II has not yet been associated with any specific disease (2). Infections with HTLV-I and HTLV-II are commonly found in certain high-risk groups, such as female prostitutes, intravenous drug users, and patients attending sexually transmitted disease clinics (2, 13). A low seroprevalence rate for HTLV-I and II (0.02%) has also been documented for normal blood donors in the United States (2).

The genomic organization of HTLV consists of structural genes \textit{(gag, pol, and env)} and a regulatory gene \textit{(pX)} at the 3' end of the genome which encodes the nonstructural proteins \textit{tax} and \textit{rex} (6, 22). \textit{Tax} is the regulatory protein of HTLV that not only \textit{trans}-activates its own long terminal repeat but also enhances transcription of several cellular genes through the activation of host transcriptional factors (4, 22). The \textit{tax} protein of HTLV-I is a 40-kDa protein (p40\textsuperscript{tax}) (22), whereas HTLV-II, which lacks the 22 amino acids (aa) at the C terminus, encodes a 38-kDa protein (p38\textsuperscript{tax}) (6). Although p40\textsuperscript{tax} is localized in the nucleus of HTLV-I-infected cells (4, 22), antibodies to p40\textsuperscript{tax} have been identified in individuals infected with HTLV-I, indicating that p40\textsuperscript{tax} is immunogenic during natural infection with this virus (21, 27). These anti-tax antibodies appear to be a marker of infectivity (12) and correlate with both mother-to-child (7, 20) and sexual (3) transmission of HTLV-I. More recently, anti-tax antibodies have also been shown to correlate with an increased HTLV-I proviral DNA load in patients with HAM (14). In addition, anti-p40\textsuperscript{tax} antibodies have been identified in relatives of ATL patients (17, 18) and in individuals who later seroconverted to HTLV-I structural proteins (5). Taken together, these findings suggest that the analysis of antibody to p40\textsuperscript{tax} of HTLV-I is important for studying both the seroepidemiology and the modes of transmission of HTLV-I. Moreover, the degree of expression of tax in vivo, as reflected in the development of antibodies to tax, may well influence the course of infection and thus play a role in disease pathogenesis.

Recently, antigenic domains of the p40\textsuperscript{tax} protein have been identified by using mouse or rat monoclonal antibodies (MAbs) specific for the tax protein (24, 25); however, the epitopes that are highly immunoreactive under natural infection have not been identified. Using overlapping synthetic peptides which span the entire p40\textsuperscript{tax}, we have identified immunodominant motifs to be located towards the amino terminus (aa 106 to 125) and at the carboxyl terminus (aa 316 to 335) of the HTLV-I tax protein (15). In the present investigation, we have used synthetic peptides representing these immunogenic regions to develop an enzyme immunoassay (EIA) which appears to be highly sensitive and type specific for the detection of anti-tax antibodies in HTLV-I-infected individuals.

MATERIALS AND METHODS

Immune reactivity to \textit{tax}. The plasmid expressing the \textit{tax} fusion protein, pTaxH\textsubscript{6}, was constructed as described previ-
ously (28). Immune reactivity to the recombinant tax (rtax) protein was determined by both enzyme immunoassay (EIA) and Western blotting (WB) (immunoblotting). For the EIA, 50 μl of purified rtax protein was coated on polystyrene plates at a concentration of 1 μg/ml in 0.1 M carbonate buffer, pH 9.6. The excess reactive sites were blocked by the addition of 5% bovine serum albumin in phosphate-buffered saline (pH 7.4) containing 0.5% Tween 20 (PBS-T) and then by the addition of a 1:50 dilution of test sera, and the plates were incubated overnight at 4°C. After six washes, a 1:1,000 dilution of alkaline phosphatase-conjugated goat anti-human immunoglobulin G (Sigma, St. Louis, Mo.) was added for 2 h at room temperature (RT); this was followed by six washes and then the addition of p-nitrophenyl phosphate (Sigma) substrate. The plates were read after 1 h with an enzyme-linked immunosorbent (ELISA) reader (SLT Lab Instrument, Ronkonkome, Austria) at 405 nm. Seropositivity was defined as any value greater than the mean of the normal controls + 2 standard deviations.

For WB analysis, rtax protein was run on 12% polyacrylamide gels (8 μg per gel) and transferred to a polyvinylidene difluoride membrane. After being blocked with 5% bovine serum albumin in PBS-T for 2 h at RT, the individual strips were incubated with a 1:100 dilution of either rabbit anti-tax antisera (obtained through the AIDS Repository) or the test serum overnight at 4°C. After three washes, the strips were incubated with a 1:1,000 dilution of goat anti-human immunoglobulin G or goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase for 2 h at RT; this was followed by color development with the 5-bromo-4-chloro-3-indolylphosphate toluidinium–nitroblue tetrazolium phosphate substrate system (Kirkegaard & Perry, Gaithersburg, Md.).

Inhibition of antibody binding to the rtax protein was carried out by adding rtax protein or synthetic tax peptide to the sera before their addition to the assay. In the EIA, diluted sera were incubated with the peptides at concentrations of 100, 10, and 1 μg per well for 2 h at RT before addition to the ELISA plate. The assay was then carried out as previously described. In the WB assay, the serum was mixed with the inhibiting antigen (rtax, 10 μg per well) for 30 min at RT before it was added to the WB strip containing the rtax antigen; then the assay proceeded as described above.

**Synthetic peptide-based immunoassay.** The peptides were synthesized by 9-fluorenyl-methoxycarbonyl chemistry, analyzed, and purified as previously described (19). The peptides analyzed from the HTLV-I sequence were Tax-8 (01-125) (LQ AMRKYSFRNQYMPEITLG), Tax-23 (16-335) (PISSLNFK fqADNDEPQQ), Tax-24 (133-350) (HEPQISPGGLEPEKH FRE), and Tax-24 (336-355) (SPGGLLEPEKHFRETEV). To determine the type specificity of these immunogenic regions, the corresponding regions from HTLV-II, Tax811 (01-125) (FQSMKHTIPYRNGCLEPTLG) and Tax22 (132-331) (YTN IPVSILFNEKEADDNGD), were also synthesized. An EIA was developed with these synthetic peptides essentially as described for rtax, except that the polystyrene plates were coated with 50 μl of Tax8, Tax22, Tax23, Tax24, Tax811, or Tax2211 peptide (10 μg/ml) either separately or in combination.

**Serum specimens.** Serum specimens from 154 individuals previously identified to be infected with HTLV-I (n = 138) or HTLV-II (n = 16) were used to analyze antibodies to tax. Serum specimens from 22 blood donors previously shown to be negative for antibodies to HTLV-I and HTLV-II were included as controls. Of the HTLV-I-infected specimens, 26 were from patients with HAM, 46 were from patients with ATL, and 66 were from asymptomatic blood donors. All of the HTLV-II specimens were from injecting drug users from the United States. All of the specimens were tested by WB to confirm the presence of antibodies to both gag and env gene products and were typed to be infected with HTLV-I or HTLV-II by the PCR or by serologic assays containing type-specific immunodominant epitopes of HTLV-I and -II, as described previously (1).

## RESULTS

**Antibodies to rtax in HTLV-positive specimens.** An EIA with the purified rtax protein demonstrated antibodies to rtax in serum specimens from 23 of 26 (88%) individuals with HAM, 11 of 46 (24%) of those with ATL, and 37 of 66 (56%) of asymptomatic HTLV-I-infected blood donors (Table 1), suggesting that antibodies to rtax could be detected in individuals infected with HTLV-I. To increase the sensitivity of anti-tax antibody detection, a WB assay was developed using this purified rtax (Fig. 1). The purity of the rtax has previously been confirmed by the detection of a major band at 40 kDa by rabbit polyclonal antisera raised against the tax protein (15). The specificity of the anti-tax antibodies was confirmed by competitive inhibition experiments, in which addition of soluble rtax protein was able to completely block the binding of antibodies to the rtax protein on the WB strip in a dose-dependent manner (data not shown). WB analysis with rtax resulted in an enhanced sensitivity of anti-tax antibody detection; 96% of the specimens (25 of 26) from HAM patients, 43% of the specimens (20 of 46) from ATL patients, and 61% of those (40 of 66) from asymptomatic carriers demonstrated anti-tax antibo-

![FIG. 1. Immune reactivity to the tax protein in patients with HAM (A), in patients with ATL (B), and in asymptomatic carriers infected with either HTLV-I (C) or HTLV-II (D). Arrow indicates the position of tax.](http://cvl.asm.org/pdf/177_A_1010.pdf)
ies (Fig. 1 and Table 1). Further, detection of anti-tax antibodies appeared to be HTLV-I type specific, since only one of the specimens from individuals infected with HTLV-II demonstrated any reactivity to the rtax in either EIA or WB (Table 1).

**Immunogenicity and type specificity of tax epitopes.** We have recently identified a major immunodominant epitope(s) (Fig. 2), located towards the amino terminus (aa 106 to 125) and at the C terminus (aa 316 to 353) of the tax protein (15). Synthetic peptides representing these epitopes from the tax protein of HTLV-I were used to determine the immunoreactivity of these regions, and corresponding regions from the HTLV-II tax proteins were analyzed to determine the viral type specificity of these epitopes. Analysis of synthetic peptides from the HTLV-I tax protein (Tax8106-125, Tax22316-335, Tax23331-350, and Tax24336-353) with 138 HTLV-I-infected specimens demonstrated antibodies in 66 (48%), 50 (36%), 66 (48%), and 64 (46%) specimens, respectively. The corresponding optical densities for each of the peptides in the different clinical groups are illustrated in Fig. 3. As expected, immune reactivities to the four peptides were highest in patients with HAM and then in asymptomatic individuals and patients with ATL. Furthermore, patients with ATL as a group revealed higher immune reactivity to Tax8 (Fig. 3B) compared with Tax22, Tax23, or Tax24 (Fig. 3E, H, and K).

Synthetic peptides from the corresponding regions of the HTLV-II tax protein were analyzed to determine the type specificity of these epitopes (as shown in Fig. 2). None of the serum specimens from HTLV-II-infected individuals demonstrated antibodies to either Tax8II106-125 or Tax22II316-331 (data not shown), suggesting that these epitopes presumably do not represent linear epitopes of the HTLV-II tax protein. To further examine the type specificity of the tax epitopes, competitive inhibition experiments were performed with se-

![Alignment of the immunodominant epitope sequences of the HTLV-I and HTLV-II tax proteins.](image)

**FIG. 2.** Alignment of the immunodominant epitope sequences of the HTLV-I and HTLV-II tax proteins. The amino acid numbering is from the amino terminus of each protein.

![Serum reactivity profiles obtained with HTLV-I sera from patients with HAM (A, D, G, and J), patients with ATL (B, E, H, and K), and asymptomatic (ASY) carriers (C, F, I, and L) against tax peptides Tax8 (A to C), Tax22 (D to F), Tax23 (G to I), and Tax24 (J to L). Each specimen is represented by an individual bar on the x axis, with the corresponding optical density shown on the y axis. n represents number of specimens tested in each group of patients.](image)

**FIG. 3.** Serum reactivity profiles obtained with HTLV-I sera from patients with HAM (A, D, G, and J), patients with ATL (B, E, H, and K), and asymptomatic (ASY) carriers (C, F, I, and L) against tax peptides Tax8 (A to C), Tax22 (D to F), Tax23 (G to I), and Tax24 (J to L). Each specimen is represented by an individual bar on the x axis, with the corresponding optical density shown on the y axis. n represents number of specimens tested in each group of patients.
Trans-Activator Protein 179

ANTIBODIES TO HTLV-1 TRANS-ACTIVATOR PROTEIN

VOLE. 1, for 2 h

Tax22, Tax23, and Tax24 was comparable to that to rtax antibody in patients with HAM (92% in the peptide assay versus 96% in the rtax WB assay) and asymptomatic carriers (56% in the peptide assay versus 61% in the rtax WB assay), peptide reactivity was lower in patients with ATL (28%) compared with the rtax WB assay (43%) (Table 1). Neither conjugation of the peptides to bovine serum albumin nor changing the molar ratio of the mixture of peptides resulted in enhanced sensitivity of detection of anti-tax antibodies (data not shown).

Since we had observed higher reactivities to Tax8 in ATL patients (Fig. 3), we next examined seroreactivity of HTLV-I specimens to Tax8 in conjunction with Tax22, Tax23, and Tax24. An equimolar ratio of these four peptides resulted in not only increased optical densities but also enhanced sensitivity of detection in all three clinical groups (Fig. 5). More importantly, the combined seroreactivity to these peptides in HAM patients (96%), ATL patients (52%), and asymptomatic carriers (62%) was even higher than that observed for the rtax WB assay (96, 43, and 61%, respectively) (Table 1). In addition, all of the specimens that reacted in the rtax WB assay also reacted with this peptide assay. Further, the antibody responses to this peptide mixture were also type specific, since none of the HTLV-II serum specimens reacted in this assay (Fig. 5).

DISCUSSION

p40tax, a product of the HTLV-1 pX gene, plays a critical role in virus replication and virus-induced lymphocyte transformation (4, 22). In addition to playing a functional role in viral transcription, tax expression appears to play an important role in viral infectivity and disease pathogenesis (12, 14). Seroopidemiological studies have further demonstrated that anti-tax antibodies are associated with an increased incidence of both vertical and sexual transmission of HTLVs (3, 7, 20).

FIG. 4. Competitive inhibition by Tax8 (■), Tax22 (▲), Tax8II (○), or Tax22II (▲) of anti-tax antibodies in serum specimens from HTLV-I-infected individuals. Peptides were incubated with test sera for 2 h at room temperature and then tested for anti-tax antibodies by EIA. Results are expressed as the mean percent inhibition of six HTLV-I-infected serum samples, including three from HAM patients and three from asymptomatic HTLV-I carriers.

The preferential immunoreactivity to synthetic peptides representing the amino terminus and the C terminus of the tax protein, together with our recent report demonstrating that a cocktail representing a mixture of two peptides from the immunodominant regions of the env protein is more sensitive for detection of anti-env antibodies than either peptide alone (19), led us to examine a combination of Tax22, Tax23, and Tax24 for anti-tax antibody detection. In general, a combination of the three peptides in equimolar ratios resulted in increased sensitivity of anti-tax antibody detection compared with any of the peptides alone. While the combined seroreactivity to Tax22, Tax23, and Tax24 was comparable to that to rtax antibody in patients with HAM (92% in the peptide assay versus 96% in the rtax WB assay) and asymptomatic carriers (56% in the peptide assay versus 61% in the rtax WB assay), peptide reactivity was lower in patients with ATL (28%) compared with the rtax WB assay (43%) (Table 1). Neither conjugation of the peptides to bovine serum albumin nor changing the molar ratio of the mixture of peptides resulted in enhanced sensitivity of detection of anti-tax antibodies (data not shown).

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FIG. 5. Serum reactivity profiles from patients with HAM or ATL and asymptomatic HTLV-I (ASY I) and HTLV-II (ASY II)-infected donors against a peptide mixture containing equimolar ratios of Tax8, Tax22, Tax23, and Tax24.
Most of the studies analyzing anti-tax responses have utilized recombinant tax protein expressed in various expression vectors in a WB format (3, 5, 12, 14, 18). However, the results of such preparations can vary depending on the concentration and purity of the recombinant protein used. Therefore, synthetic peptide-based assays provide an attractive alternative to the recombinant WB assay in view of their reproducibility and cost-effectiveness. In the present investigation, we have used immunodominant epitopes from p40\textsuperscript{tax} to develop a highly sensitive synthetic peptide assay that allows rapid detection of anti-tax antibodies in HTLV-I-infected individuals.

The relative antibody reactivities to the purified tax seemed to be higher with denatured forms of the antigen in the WB format (62%) than with non-denatured forms in the ELA (51%). The chemical denaturation of the protein might expose certain epitopes not available when ELA plates are coated. Alternatively, the reactivities to tax in serum specimens from HTLV-I-infected individuals may be conformation dependent. Further analysis of antibody reactivities in clinical groups revealed the highest reactivities in HAM patients (96%) compared with asymptomatic carriers (61%) and ATL patients (43%). These results are in general agreement with previous reports in which almost all of HAM and approximately 50% of ATL and asymptomatic donors have been shown to contain anti-tax antibodies (11, 27). The increased incidence of anti-tax antibodies in HAM patients could be attributed to altered immune responses in this group of patients (8). Indeed, polyclonal B-cell activation, a spontaneous state of T-cell activation, and activated cytotoxic T-cell activity have been shown to react with 43% of serum specimens from individuals infected with HTLV-I. The carboxyl terminus of the tax protein contains a linear epitope representing an immunodominant epitope of the tax protein.

The immunogenicity of the carboxyl-terminal domain of the tax protein has previously been identified by virtue of antisera to the C-terminus peptide or MAb detecting epitopes at the C terminus of the tax protein (24, 25). In fact, most MAb to the p40\textsuperscript{tax} protein have identified two nonoverlapping epitopes at the C terminus of the tax protein: MAb TAXY-7 reacts with an antigenic determinant between aa 286 and 338, whereas TAXY-6 and TAXY-8 react with an epitope at aa 339 to 353 (24). Our results further demonstrate that the synthetic peptides Tax22\textsuperscript{316-335}, Tax23\textsuperscript{331-350}, and Tax24\textsuperscript{336-353} represent immunodominant motifs for the anti-tax antibodies in individuals infected with HTLV-I.

It is of interest that only one of the serum specimens from individuals infected with HTLV-II reacted with the tax protein of HTLV-I. This suggests that the two tax antigens share little antigenic homology, although the p40\textsuperscript{tax} and p38\textsuperscript{tax} proteins share significant homologies (about 75%) at the nucleotide sequence level (6, 23). However, the sequence analysis demonstrated that the HTLV-II tax contains a stop codon which results in a protein that is 22 aa shorter than its counterpart HTLV-I tax protein (6, 22). The fact that the C terminus of p40\textsuperscript{tax} contains the immunodominant epitope(s) and that the p38\textsuperscript{tax} lacks this motif may account for the type-specific responses to the tax protein. In addition, the C terminus of the HTLV-II tax protein does not appear to contain a linear epitope that elicits an antibody response, since synthetic peptide (Tax22\textsuperscript{212-331}) representing the C-terminus of truncated p38\textsuperscript{tax} was not immunoreactive with serum specimens from individuals infected with HTLV-II. The type specificity of the HTLV-I tax protein is further confirmed by recent studies in which none of the rat or mouse MAb was shown to react with the p38\textsuperscript{tax} protein (24, 25).

Since multiple antigenic epitopes on a protein can lead to polyclonal antibody responses in infected individuals, we argued that multiple peptides representing these epitopes might result in enhanced sensitivity of antibody detection. Indeed, a combination of the peptides (Tax8, Tax22, Tax23, and Tax24) in equimolar ratios was highly sensitive in detecting anti-tax antibodies in 96% of patients with HAM, 52% of those with ATL, and 62% of asymptomatic blood donors. More importantly, the results obtained by this peptide cocktail assay were comparable to those obtained by the tax WB assay. In fact, the peptide cocktail assay also detected a few additional specimens that were missed by the WB assay. The type specificity of the peptide assay was further confirmed by the absence of any reactivity with serum specimens from infected individuals with HTLV-II.

In summary, we have developed a synthetic peptide-based assay that provides a simple tool to analyze anti-tax antibodies in individuals infected with HTLV-I. The assay is capable of detecting antibodies in all individuals in whom anti-tax was detected by the WB assay and is type specific for HTLV-I infection. Thus, the assay provides a simple format to analyze anti-tax antibodies for seroepidemiologic studies to determine markers of infectivity and also for intrahalal studies to determine tax antibodies among blood relatives of ATL patients.

REFERENCES