Detection of Anti-HTLV-I Tax Antibodies in HTLV-I Enzyme-Linked Immunosorbent Assay-Negative Individuals


The HTLV-I tax gene protein (Tax) is not packaged within the mature viral particle from which the proteins for the commercially available enzyme-linked immunosorbent assay (ELISA) are derived. Screening of 162 individuals within a cohort of white intravenous (IV) drug abusers, previously identified as having an increased incidence of HTLV-I infection, demonstrated that seven of them had antibodies to the HTLV-I Tax protein but tested negative in HTLV-I ELISAs and Western blots prepared from purified virion proteins. Three out of 35 individuals in other behaviorally defined high-risk groups also displayed this limited pattern of reactivity to HTLV-I proteins. The presence of the anti-HTLV-I p40/Tax antibodies was determined by radioimmunoprecipitation assay (RIPA), which also revealed low levels of anti-env reactivity. The specificity of the anti-p40 reactivity was confirmed on specific Tax antigens and the HTLV-I nuclear protein, p40/Tax.

HTLV-I and the related viruses HTLV-II, -V, bovine leukemia virus (BLV), and simian T-cell leukemia virus (STLV) are distinguished from other mammalian type C oncoviruses by the possession of a 3' gene, tax, which encodes a transcriptional transactivator protein that positively regulates the HTLV-I viral transcriptional unit. The principal location of the tax gene protein (Tax) is the cellular nucleus rather than the viral particle.

In asymptomatic HTLV-I carriers and HAM patients (ie, those who do not have a lymphocytosis), the provirus is often undetectable in fresh peripheral-blood mononuclear cells (PBMC) by standard Southern hybridization techniques. This problem has recently been overcome by use of the enzymatic in vitro gene amplification technique (PCR), which can amplify a sequence of interest more than a millionfold.

METHODS

Patients. Serum from 162 intravenous (IV) drug abusers (IVDA) and 35 individuals in other retroviral risk groups (offspring of IVDA; heterosexual contacts with IVDA; homosexuals, and a man who had lived and traveled in malarial Africa) were analyzed for their specific reactivities against the various HTLV-I viral antigens and the HTLV-I nuclear protein, p40/Tax. These sera had been previously collected as part of a prospective ELISA screen for anti-HTLV-I antibodies. All of the patients were from Staten Island, NY, and all were asymptomatic for lymphoproliferative or neurologic abnormalities associated with HTLV-I. Three percent (6/197) of these serum samples had been previously identified by ELISA and confirmed by RIPA and/or Western blot as being anti-HTLV-I positive.

Assays to detect HTLV-I antigens. Anti-Tax hyperimmune serum was obtained by immunizing and repeatedly boosting a New Zealand white rabbit with recombinantly produced Tax protein purified from Escherichia coli. The serum from this rabbit was used to compare the levels of Tax protein in the various HTLV-I serologic assays. Anti-HTLV-I viral protein hyperimmune serum was obtained in a like manner using detergent-disrupted HTLV-I virions that had been purified by repeat bandings on sucrose gradients. This serum was used to assess the levels of the various HTLV-I gag- and env-encoded peptides in several serologic assays. Serum from an unimmunized rabbit was also used in these assays.

Assays for antiretroviral antibodies. HTLV-I RIPA were per-
formed as previously described,21 except that in some cases two identical mixed cultures of SLB-I and MT2 cells were metabolically labeled with 35S methionine and 35S cysteine, respectively, for four hours. These two cultures were then combined to prepare a doubly labeled mixed lysate to ensure high levels of all HTLV-I proteins. HIV-1 ELISA and HTLV-I ELISA and Western blot kits were provided by Cellular Products Inc (Buffalo, NY) and used according to the manufacturer's specifications. HTLV-II ELISAs were performed exactly as described,26 except that HTLV-II virion proteins from banded particles produced by the MOT cell line were used as the source of antigen. The HTLV-I tax protein ELISA has been previously described.36 Briefly, assays were performed by incubating 1:20 dilutions of serum samples for one hour at 37°C in duplicate microtiter wells coated with 200 ng of purified recombinant HTLV-I tax protein. After rinsing with phosphate-buffered saline (PBS)/0.05% Tween 20, bound human antibody was detected with protein A conjugated to horseradish peroxidase and o-phenylenediamine/H2O2. The reaction was measured at 490 nm with a Dynatech MR700 microplate reader. Tax Western blots were prepared by electrophoresing purified recombinant tax protein through a Laemmli gel system and transferring the protein to nitrocellulose by electroblotting. The filter was washed for 15 minutes in 1X PBS (GIBCO, Grand Island, NY), 0.3% Tween 20, and stored on 3MM Whatman paper in the dark. Strips containing 70 ng of protein were cut from the filter (∼50-mm length × 3-mm width). Individual strips were placed in polypropylene tubes and blocked for one hour with 1X PBS, 0.3% Tween 20, 3% bovine serum albumin (BSA) on a rocking platform at 24°C, followed by washing three times with 1X PBS, 0.3% Tween 20. Serum samples were diluted 1:50 in wash solution, added to the filter strips, and incubated overnight on a shaker at 4°C. Filters were then washed three times as above. Amplified DNAs were assayed in spot-blot formats as described.7 The regions of the amplified DNAs were assayed in spot-blot formats as described. The sensitivity and specificity of the HTLV-I ELISA, and Western blot assays prepared from viral proteins, and the HTLV-I RIPA based on infected cell lysates have been previously established26 (Poiesz et al; Anderson et al; and Slamon et al, manuscripts in preparation). The validity of the recombinant Tax ELISA and a cut-off determination were established by running 187 normal human serum samples and measuring the resultant absorbances. The mean of these samples was determined to be 0.126 with an SD of 0.073. Using the same convention that we employ for the HTLV-I virion protein ELISA, where the cut off is defined as the mean of the normals plus 5 SDs, a cut-off value of ∼0.500 was established. The specificity and sensitivity of the Tax ELISA were further investigated in a double-blind analysis in which samples from SUNY HSC were coded and sent to Triton Biosciences Inc. The mean absorbance readings for various groups were as follows: ATL and HTLV-I associated myelopathy (HAM) patients 2.000 (four had absorbance readings between 1.523 and 1.997; 16 were >2.000), n = 20; random Red Cross blood donors 0.155, n = 60; other diseases, including acquired immunodeficiency syndrome (AIDS), lymphoma, granulomatous syndromes, autoimmune diseases 0.187, n = 90. A single patient with an HTLV-I benign lymphoproliferative disease gave an absorbance reading of 1.090. These findings established the sensitivity and specificity of the Tax ELISA to be 100% and 99.1% (3/337 normals and individuals with other diseases tested above the cutoff), respectively. All patients with clinically expressed HTLV-I disease tested in this study were positive in the Tax ELISA.

The HTLV-I virion protein ELISA was the least sensitive assay of these methods for the detection of immunoreactivity directed against the HTLV-I tax gene protein (Table 1). The HTLV-I RIPA was approximately 50 times more sensitive, and the specific assays (ELISA and Western blot using recombinantly prepared Tax) designed to specifically detect seroreactivity to Tax were approximately three orders of magnitude better than the standard HTLV-I ELISA. A

Table 1. Comparison of Various Serologic Assays for Their Ability to Detect Antibodies Against HTLV-I Viral Proteins and Tax

<table>
<thead>
<tr>
<th>Assay Serum</th>
<th>HTLV-I RIPA* 40-Kd Tax</th>
<th>Tax† Western</th>
<th>HTLV-I† ELISA</th>
<th>HTLV-I‡ Western</th>
</tr>
</thead>
<tbody>
<tr>
<td>αTax rabbit</td>
<td>5,000</td>
<td>&gt;100,000</td>
<td>90</td>
<td>500</td>
</tr>
<tr>
<td>Normal rabbit</td>
<td>0</td>
<td>500</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>αHTLV-I rabbit</td>
<td>0</td>
<td>500</td>
<td>9,000</td>
<td>ND</td>
</tr>
<tr>
<td>HAM patient</td>
<td>1,000</td>
<td>8,000</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Laboratory worker</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

All data are given as the reciprocals of the highest dilution at which the serum gave a positive signal.

Abbreviation: ND, not determined.

*End points for RIPAs were determined by assessing the highest serum dilution at which a 40-Kd band could be visualized by fluorography. Sera were run at 2 × 10−4, 10−3, 2 × 10−3, 10−2, and 10−1 dilutions.

†End points for ELISAs were determined by plotting the ODmax results versus serum dilution and determining where the individual curves crossed the cut-off line, defined as 5 SDs above the mean of the normal control samples. Sera were run at 2 × 10−2, 10−1, 10−2, 10−3, 10−4, and 10−5 for the tax protein ELISA and at 2 × 10−2, 10−1, 10−2, 10−3, 10−4, and 10−5 for the HTLV-I ELISA.

‡End points for all Western blots were determined by assessing the highest serum dilution at which a 40-Kd band could be visualized following color development. Sera were run at 2 × 10−2, 10−1, 2 × 10−2, 10−3, 10−4, and 10−5 dilutions.

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comparison of the results obtained with the normal rabbit serum and those from the hyperimmune anti-Tax rabbit indicate that there is a minimal amount of Tax in the virion-prepared ELISA and Western blot. The normal rabbit serum used in this comparison, however, contains limited Tax reactivity as it is presented in the Tax immunoblot, which probably explains the reactivity exhibited by the hyperimmune anti-HTLV-I viral protein serum. Neither of these sera react in the Tax-specific ELISA, nor do they precipitate Tax in the RIPA, although the gag and env proteins are detectable at titers of 1:1,000 when the HTLV-I hyperimmune rabbit’s serum is employed. The normal human sera tested did not react in any of the HTLV-I antibody assays. Serum from a HAM patient, previously demonstrated by RIPA to contain reactivity to all HTLV-I Tax reactivity as it is presented in the Tax immunoblot, used in this comparison, however, contains limited serum antibodies these sera react in the Tax-specific ELISA, nor do they which probably explains the reactivity exhibited by the SLB-I.

Cell line, T-cell lymphoma sent anti-Tax antibodies. None of these sera immunoprecipitated a 40-Kd band from an HTLV-I negative cutaneous T-cell lymphoma cell line, HUT 78. There were no significant bands corresponding to the viral gag (p24) and gag precursor (p55) proteins of HTLV-I in 8/10 of these sera; the remaining two had weak reactivity against both gag proteins, as opposed to the ELISA-positive ATL and IVDA patient sera which were strongly reactive against these gag antigens (Fig 1). These ten patients, testing positive only by RIPA, did demonstrate weak-to-moderate reactivity against the HTLV-I envelope glycoproteins gp68 and/or gp46. Using sera from HTLV-I-infected patients, a comparison of anti-env signal intensity in HTLV-I RIPAs and Western blots demonstrates that the envelope proteins tend to be under-represented in relation to the gag proteins of HTLV-I in the viral preparations used for the ELISA and Western blot assays.12 This observation helps explain the lack of positive ELISA results among these individuals who display predominantly anti-Tax and anti-env reactivity in the RIPA.

Due to the fact that there exists substantial homology between the tax genes of HTLV-I and II, the possibility existed that these patients were infected with HTLV-II. Assessment of these sera in our HTLV-II viral protein ELISA revealed them all to be negative for anti–HTLV-II antibodies (Table 2).

Eight of ten sera samples that were unreactive in the HTLV-I ELISA but that immunoprecipitated a 40-Kd protein from lysates of an HTLV-I–infected cell line (SLB-1) were confirmed as having specific anti-Tax antibodies in the recombinant Tax ELISA. The remaining two samples assayed in this format gave readings substantially above most normals (OD > 0.350) but below our stringent cutoff (Table 2). Sera from all ten of these individuals were again negative in an HTLV-I viral-protein ELISA run simultaneously. Further confirmation of the specificity of the anti-40 Kd/Tax ELISA reactivities was obtained by testing the sera from these ten patients in a Western blot assay using recombinant Tax as the sole antigenic source. Sera from all ten risk-group individuals as well as ATL patients demonstrated specific anti-Tax reactivity in this format, but normal control sera did not (Fig 2).

In the case of four of these RIPA +/ELISA – patients, we were able to obtain mononuclear cells from which DNA was isolated. The DNA was gene amplified by PCR for the HTLV-I pol and tax genes, with primer pairs SK54/55 and SK43/44, respectively. In all four of these patients, HTLV-I homologous sequences were detected by this methodology (Fig 3). DNA samples from all four patients were repeatedly positive with the tax primer-detector system when assayed by spot-blot hybridization (Fig 3A). In addition, three of four samples were positive in the higher stringency liquid hybridization (LH) assay (Fig 3B). One of the patients (5044) from whom two separate DNA isolations were available was repeatedly positive in the tax spot-blot hybridization assay, but only the earlier sample was LH.

### Table 2. Serum Reactivities of Retroviral Risk-Group Members

<table>
<thead>
<tr>
<th>Patient</th>
<th>HTLV-I Tax ELISA</th>
<th>HTLV-I Tax Western</th>
<th>Tax ELISA</th>
<th>Tax Western</th>
</tr>
</thead>
<tbody>
<tr>
<td>5044</td>
<td>19 (–)</td>
<td>–</td>
<td>952 (+)</td>
<td></td>
</tr>
<tr>
<td>5048</td>
<td>177 (–)</td>
<td>–</td>
<td>&gt;2,000 (+)</td>
<td></td>
</tr>
<tr>
<td>5079</td>
<td>88 (–)</td>
<td>–</td>
<td>727 (+)</td>
<td></td>
</tr>
<tr>
<td>5173</td>
<td>36 (–)</td>
<td>–</td>
<td>1,308 (+)</td>
<td></td>
</tr>
<tr>
<td>5324</td>
<td>142 (–)</td>
<td>–</td>
<td>998 (+)</td>
<td></td>
</tr>
<tr>
<td>5327</td>
<td>32 (–)</td>
<td>–</td>
<td>1,714 (+)</td>
<td></td>
</tr>
<tr>
<td>5334</td>
<td>49 (–)</td>
<td>–</td>
<td>1,564 (+)</td>
<td></td>
</tr>
<tr>
<td>5351</td>
<td>56 (–)</td>
<td>ND</td>
<td>533 (+)</td>
<td></td>
</tr>
<tr>
<td>5354</td>
<td>53 (–)</td>
<td>ND</td>
<td>393 (+/-)</td>
<td></td>
</tr>
<tr>
<td>5370</td>
<td>32 (–)</td>
<td>ND</td>
<td>314 (+/-)</td>
<td></td>
</tr>
</tbody>
</table>

All serum samples analyzed were identified in a HTLV-I RIPA as having antibodies against a 40-Kd protein present in cellular lysates prepared from SLB-1.

Abbreviations: MA, malarial Africa; HS, homosexual; F1, offspring of risk group member; W, white; B, black; ND, not determined.
Fig 1. HTLV-I RIPAs of serum samples from patients with adult T-cell leukemia and IVDAs. RIPAs were performed as described in Methods with SLB-1 cell lysates that were metabolically labeled with $^{35}$S methionine and cysteine. For both panels the migrations of the mol wt markers are given on the left-hand side, and specific HTLV-I viral antigens are indicated on the right-hand side. Serum samples are as follows: (A) lane 1, ATL patient; lane 2, laboratory worker; lanes 3 to 6, ATL patients; lane 7, random Red Cross blood donor. (B) lanes 1 and 2, HTLV-I virion protein ELISA and Western blot-positive IVDAs; lanes 3 to 7, HTLV-I virion protein ELISA and Western blot-negative IVDAs patients 5044, 5048, 5079, 5173, and 5327, respectively.

**DISCUSSION**

A serologic analysis had been previously conducted to determine the extent of exposure to HTLV-I among a cohort of primarily white, US born IVDAs from the New York City area. Six of 162 (3.7%) patients, all white, were found to be repeatedly reactive in an HTLV-I ELISA prepared from viral proteins. All of these HTLV-I-positive results were confirmed by RIPA and/or Western blot as having antibodies directed against HTLV-I gag, env, and tax gene products.

In the present study we describe a phenomenon of limited reactivity to HTLV-I proteins in 7/162 individuals from this IVDA cohort and 3/35 individuals from other risk groups. When these results are combined with the initial survey, it brings the total number of HTLV-I seroreactive individuals in the IVDA cohort to 13/162 (8.0%), thereby more than doubling the number of suspected HTLV-I-infected individuals. These ten patients whose sera were negative in a commercial HTLV-I ELISA displayed strong anti-Tax and moderate anti-env reactivity by RIPA but very little if any anti-gag reactivity. These findings, when combined with the knowledge that shearing of the HTLV-I envelope proteins occurs during gradient purification, indicate that the ELISAs presently available contain predominantly gag-encoded epitopes with lower levels of env proteins and almost no Tax.

The detection of HTLV-I homologous sequences in the DNA of all four of the RIPA+/ELISA− individuals from whom cells were available suggests that a majority of the patients identified by RIPA as having predominantly anti-tax antibodies also carry the HTLV-I provirus. The provirus from the patient who was LH negative for tax probably contains a small mutation that affects oligonucleotide hybridization, but the seroreactivity data indicate that the gene is transcribed. The patient (5044) displaying the variable tax gene LH results between samples represents an interesting case. Reamplification and detection of both samples with both assay formats confirmed the initial observa-
Fig 2. HTLV-I tax protein Western blot. Serum samples are as follows: lane 1, anti-tax protein hyperimmune rabbit; lane 2, unimmunized rabbit; lane 3, normal human; lane 4, HAM patient; lane 5, normal human; lanes 6 to 15, HTLV-I virion protein ELISA negative, RIPA-positive (40-Kd band) retroviral risk-group members. Patients 5044, 5048, 5074, 5173, 5324, 5327, 5334, 5354, 5370, and 5351, respectively.

Fig 3. In vitro enzymatic gene amplification of patient DNA samples by the PCR. Specific amplification was supported for HTLV-I tax or pol gene regions using primer pairs, SK43/44 and SK54/55, respectively. The amplified DNAs were analyzed with end-labeled oligonucleotide probes [SK45 and SK56, respectively] in two assay systems: a moderate stringency spot-blot hybridization method (1.08 mol/L NaCl; 55°C; A); or a higher stringency liquid-hybridization format (0.15 mol/L NaCl; 55°C). followed by polyacrylamide gel electrophoresis and autoradiography (B and C). (A) HTLV-I tax gene amplification, spot-blot hybridization analysis: lanes 1 to 5 represent patients 5048, 5044, 5327, 5351, and 5044 (sample 2), respectively; lane 6, adult T-cell leukemia/lymphoma (ATL) patient's DNA; lane 7, negative control DNA. (B) HTLV-I tax gene amplification liquid hybridization analysis: lane 1, negative control DNA; lanes 2 to 4, respectively, 10⁻⁶, 10⁻⁸, and 5 x 10⁻⁹ μg of an adult T-cell leukemia/lymphoma patient's DNA; lanes 5 to 9, respectively, patients 5048, 5044 (sample 1), 5327, 5351, and 5044 (sample 2). (C) HTLV-I pol gene amplification: lane 1, negative-control DNA; lanes 2 to 4, respectively, 5 x 10⁻⁶, 10⁻⁸, and 10⁻⁴ μg of an adult T-cell leukemia/lymphoma patient's DNA; lanes 5 to 9, patients 5048, 5044 (sample 1), 5327, 5351, and 5044 (sample 2).
phenomenon could be reflective of a normal expression pattern of the provirus. The phenomenon of having antibodies to regulatory proteins only may be reflective of the expression pattern of proviruses early after infection. It has been recently reported that some asymptomatic HIV patients who are HIV ELISA negative have antibodies against the regulatory nef protein (Dr C. Baechot, World Health Organization Meeting, November 7-11, 1988, Paris). It is also quite possible that certain individuals do not, for one reason or another, develop an immune response to the gag proteins. It is highly unlikely, based on the compartmentalization of the Tax protein and the virus’s life cycle, that these individuals did not have or do not have a productive infection.

It is formally possible that the PCR pol-negative patient is infected with a virus related to HTLV-I. If this is the case, however, it is not HTLV-II because this patient, as well as all the other patients with only anti-tax protein reactivity, was unreactive in an HTLV-II virus-specific ELISA. This patient was also negative in an HTLV-II pol-specific amplification assay. His DNA was amplified with the HTLV-II-specific pol gene primers, SK58/59, and the amplified DNA was analyzed by liquid hybridization with SK60. We are currently attempting to follow up on these patients as well as the remaining anti-tax-positive patients.

The data reported herein double the confirmed exposure rate to HTLV-I within a cohort of white IDVAs and indicates that this phenomenon can occur in other at-risk populations. None of the ten sera were reactive in HTLV-I ELISAs prepared from viral proteins. These findings have important implications from both a public health perspective and for analyses of HTLV-I viral pathogenesis. We have developed a new ELISA that contains HTLV-I viral antigens and recombinant Tax protein. In preliminary trials this assay has produced a positive signal in a number of individuals in high-risk groups who were negative by the standard HTLV-I viral protein ELISA.

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