Unusual Pattern of Antibodies to Human T-Cell Leukemia Virus Type-I in Family Members of Adult T-Cell Leukemia Patients


Detection methods for the human T-cell leukemia virus type-I (HTLV-II) for blood screening and diagnosis generally rely on antibody tests that use the structural proteins of HTLV-I as antigen. We have found an unusual pattern of antibody reactivity among people who are at high risk of HTLV infection due to being a family member of an adult T-cell leukemia (ATL) patient: a specific antibody reaction exclusively directed to the HTLV regulatory protein tax, and not to the HTLV-I structural proteins. Sera from 7 of 82 (8.5%) structural antibody-undetectable family members of ATL patients had the anti-tax reactivity. Two seroconverters were observed. One seroconverter, a healthy resident of Miyazaki, tested negative for structural antibody, but positive for tax antibody. Two years later she tested positive for both. The other seroconverter, an Israeli hemophiliac, tested negative for both antibodies, but converted to tax antibody-positive/structural antibody-negative. The HTLV-I tax-only antibody profile was also observed in sera sets from two other populations at risk for HTLV infection, human immunodeficiency virus-1-infected patients at the Bronx-Lebanon Hospital in New York and Israeli hemophiliacs. DNA samples from lymphocytes of four individuals with antibody reactivity only to HTLV-I tax were tested in polymerase chain reaction experiments; no HTLV-I or -II DNA was detected.

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Materials and Methods

Blood samples. Serum samples were collected from 141 family members of ATL patients presenting at the Miyazaki Medical School, Miyazaki, Japan. Whole blood was available from 50 of the family members and peripheral blood mononuclear cells (PBMCs) were purified by Ficoll-Hypaque separation, washed twice in phosphate-buffered saline (PBS) and stored as pellets at −70°C for DNA purification. Most of the family members are children, siblings, or grandchildren of ATL patients. Serum samples from 988 residents in the Miyazaki Prefecture who are involved in the Miyazaki follow-up study were also tested.56 Sixty-six sera from a group of Israeli hemophiliacs and 240 sera from the Bronx-Lebanon Hospital human immunodeficiency (HIV) clinic were screened, and one individual from each of these groups that only had antibodies against tax was available for an additional blood test to obtain PBMCs for DNA purification. One hundred eighteen baboon sera from the Southwest Regional Primate Center were screened, and a second blood sample was collected from a set of six baboons to obtain PBMCs.

Serologic assays for HTLV-I-reactive antibodies. Japanese sera were initially tested by the PA assay (Fujirebio, Tokyo, Japan) and r-tax WB assay.39 PA-negative, r-tax WB-positive samples were further tested by fluorescent antibody (FA) using HTLV-I-positive HUT-102 cells, and two ELISA assays (Abbott Lab, North Chicago, IL, and Cambridge BioTech Corp, Worcester, MA). Positive samples were also tested by WB using MT-2 cells as a source of antibody.
antigen, and RIP23 using 35S-labeled HUT-102 cells1 and C81/66 cells.24 Sera from the Bronx-Lebanon Hospital were initially screened with r-tax WB and Abbott ELISA. The serostatus of ELISA-negative, tax-positive samples was confirmed with HUT-102 RIP and WB.

All baboon sera were tested with r-tax WB and HUT-102 WB. Positive samples and representative negative controls were tested by HUT-102 RIP, as well as Cambridge BioTech and Abbott ELISAs.

**DNA extraction.** PBMCs were separated by Ficoll-Hypaque density gradient and washed twice in PBS. The cell pellets were either stored at -70°C or lysed immediately by sodium dodecyl sulfate (SDS)/protease K treatment at 56°C. The DNA was phenol extracted and ethanol precipitated, resuspended in 10 mmol/L Tris HCl, 1 mmol/L EDTA (TE) and the concentration was determined by a spectrophotometric reading at 260 nm.25 All extractions of DNA samples were accompanied by extraction of DNA from two to five tubes of blood provided by a blood donor center in Boston, which served as negative controls for polymersase chain reaction (PCR). DNA was extracted in a tissue culture facility in a separate area from where PCR products were analyzed. Strict precautions were maintained to avoid contamination with PCR product in the areas where DNA was processed or PCR reactions were set up.

**PCR.** All DNA samples were tested with several different primer pairs that can amplify HTLV-I or HTLV-II, and with primers that amplify β-globin to verify that the extracted DNA was able to serve as a good template for PCR. Two micrograms of PBMC DNA were typically used per reaction (there are approximately 140,000 human genome equivalents in 1 μg of DNA). Each experiment included two to five negative control DNAs from blood donors that had been coextracted with the samples, a negative control using DNA from the uninfected cell line HUT-78, and a reaction with cocktail-only, and no added DNA. In addition, a 10-fold dilution series was included in each reaction set using serial dilutions of HTLV-I-infected HUT-102 cells diluted in a background of 2 μg of uninfected HUT-78 DNA to determine the sensitivity of each experiment. (The number of HTLV-I genomes per cell in our HUT-102 culture was determined by comparing the intensity of PCR amplification product signals from serial dilutions of HUT-102 cells with dilutions of a known number of molecules of the HTLV-bearing plasmid pMT2 that was cut with the restriction enzyme Pst I. We estimated that there were seven HTLV-I genomes per HUT-102 cell.) The MgCl2 and primer concentrations were optimized for each primer pair. Amplification reactions contained template DNA, 1.25 mmol/L of each dNTP, 50 mmol/L KCl, 10 mmol/L Tris HCl, pH 8.3, 2.5 to 4 mmol/L MgCl2, and 0.2 to 1 μmol/L of each primer in 100 μL total volume. Reactions were heated to 95°C for 3 minutes, then cycled 39 times for 1 minute at 95°C, 45 seconds at 55°C, and 30 seconds at 72°C using an MJ Research thermocycler (MJ Research, Inc, Watertown, MA). The primer pairs had a range of different sensitivities, even when optimized. All PCR experiments were assayed by viewing the PCR product in the areas where DNA was processed or PCR reactions were set up.

All primers gave a strong distinct band of the appropriate molecular weight on an ethidium-stained gel that reacted specifically with the appropriate oligoprobe when analyzed by Southern blot,26 using HUT-102 (HTLV-I-infected) cells DNA or C344 (HTLV-II-infected cells) DNA templates as appropriate. The sequence of oligonucleotides used and the bases that define the outer boundaries of the amplified region are shown. The base numbers and sequences correspond to the sequences in Genbank, accession numbers J02029 for HTLV-I, and M10060 for HTLV-II. Primers SK43 and SK44 amplify both HTLV-I and II tax region DNA, and were probed with SK45.27 SG231 and SG238 amplify from the HTLV-I pol region, and were probed with SG232.28 RT79 and RS80 amplify DNA from the β-globin gene and were used as controls.29 Primers RM3 and RM4 amplify from the HTLV-I LTR (bases 420-720) and were probed with RM3/4: RM3, GGGCTTACCCGCCCCTGATCGCTCTC; RM4, CCAACGGATGTCGCCGACCTGGCTACCTGGG; RM3/4, GTCCCTCTGAACTGCTGCGCCTGGGTACTTTAG; Primers BKgl and BKg2 amplify from the HTLV-I gag region (bases 660-910) and were probed with RM4: BKgl, CACCTTCTACTTCAGTACTGCTGCT; BKg2, CTGAGGAAGTTAAGCCGATGTGAGCGCCG. All primers RM1 and RM2alt amplify from the HTLV-II env region (bases 5452-5726) and were probed with RM1/2: RM1, GGGGCTAATGCTAATCAGGATTGGT; RM2alt, AAGTTGAGGACGGGTAGGTTACCGTT; RM1/2, CAGCCAAACTTCTGAACTCCAGGAGGG; Primers BKprio1 and BKprio2 amplify from the HTLV-I protease region (bases 2180-2380) and were probed with BKprio1/2: BKprio1, CATTAGATCCCCGCGCTG-GCGGCG; BKprio2, GAGGTGAGCCTAAGTACCTTG; BKprio1/2, GTTCCTAAGTAAACTCCCTACCAAAATACCTGG-TATTAGG.

**RESULTS**

**Seroanalysis of family members of ATL patients.** Fifty-nine (41.8%) of 141 family members of ATL patients tested HTLV-I antibody-positive by the PA and IF methods. At the same time, all 141 family members were tested by r-tax WB. Of the 82 PA- and IF-negative sera, seven (5.8%) tested HTLV-I tax antibody-positive. These seven samples also tested negative by two ELISA kits (Abbott Labs and Cambridge BioTech Corp). The people with this antibody profile have an immediate family member (parent or sibling) who tested positive for HTLV-I antibodies.

The seven samples were further tested by RIP using HUT-102 cells (Fig 1). No reactivity to gag or env products was detected, although an antibody response to p42 tax was observed. This finding concurred with the positive signals from the r-tax WB assay. One of the seven samples (no. 114) was also tested by RIP using C81/66 cells, a cell line known to express only the HTLV-I p42 tax protein (not the structural proteins) at a detectable level. Sample no. 114 also reacted with p42 tax from this cell line. All seven samples were tested by WB using MT2 cells (data not shown). They did not show any reactivity to gag (p28, p24, and p19) or env proteins (gp61 and gp45). The tax protein cannot be detected by conventional WB assay.

**Serum samples from the Miyazaki follow-up study.** Of 988 serum samples tested by PA assay, 254 were positive for
Seroanalysis of additional groups at risk for HTLV infection.
To confirm that antibody reactivity specific to the HTLV tax protein existed in groups beyond the population in Southern Japan, and to extend our pool of people with this serologic profile, we screened our sera collection for the presence of antibodies specific to HTLV tax (not to structural proteins) from two additional sources: an HIV-infected population in the South Bronx and Israeli hemophiliacs. 125 HIV+ sera and 115 HIV- sera from the Bronx-Lebanon Hospital were screened by r-tax WB and

HTLV-I antibodies (25.7%). Of the 734 PA-negative samples, 11 (1.5%) tested positive by r-tax WB. They were also confirmed to react with tax protein, p42, but not with the viral structural proteins gp61, gp45, p28, p24, and p19 by RIP using HUT-102 cells.

Sequential samples were available from one of the 11 people, a 70-year-old woman who seroconverted from tax-only status to structural antibody-positive. Serum from blood drawn in 1984 was negative by PA but positive by r-tax WB. Sera taken in 1986 and 1987 were positive both by PA and r-tax WB. An RIP assay was performed on the sequential serum samples, and reactivity to p42-tax but no other viral protein was detected in the 1984 sample, with an additional gp61-env reactivity apparent in the later samples (Fig 2). Lymphocytes were available from the blood test in 1990 after her seroconversion to gp61-positive, at which point she was PCR-positive (data not shown).
Abbott HTLV ELISA. Of the 125 HIV⁺, 11 (8.8%) were dual positive for HTLV and HIV, and four of 114 (3.5%) HTLV ELISA-negative samples were tax WB-positive. Of the 115 HIV⁻, two (1.7%) were positive by HTLV Abbott ELISA, and two of 113 (1.8%) ELISA-negative samples were positive only by tax WB. The tax-only serostatus was confirmed by HUT-102 WB and RIP (data not shown). The HTLV antibody status was similarly checked in a group of 66 Israeli hemophiliacs.³⁰ Three of 66 (4.5%) had antibodies to HTLV structural proteins. A single individual in this group expressed antibodies to tax-only. Serial samples were tested using HUT-102 RIP from this individual. He seroconverted from HTLV-negative to HTLV tax-only, remaining positive for HTLV tax-only for a period of 10 years (data not shown).

Specificity of the anti-tax antibody response. The fact that sera that react with the 50-Kd bacterial recombinant tax fusion protein also have an antibody against the viral p42 protein when assayed by RIP indicates that the reaction is specific. Furthermore, the recombinant tax-ras fusion protein used in our tax WB is able to specifically block the anti-p42 tax reactivity in an HUT-102 RIP, when the recombinant tax protein is present in excess (Fig 3). A recombinant ras peptide that represents the ras portion of the ras-tax fusion protein does not have this capacity (Fig 3).

PCR analysis of PBMCs. Lymphocytes were available from a limited number of people in this study. Therefore, PCR testing was performed on a total of four individuals with antibodies specific to tax and not to HTLV structural proteins. Lymphocytes from 48 ATL family members were available for PCR analysis. These included 14 healthy carriers defined by the usual criterion of antibodies to structural proteins, 32 seronegatives, and two people with antibodies specific to tax. Additional seronegative controls were included during DNA processing using blood samples from healthy blood donors at the Children’s Hospital in Boston. DNA derived from PBMCs of five healthy seropositive blood donors from Miyazaki was used to test additional positive controls. PBMCs were also available from one individual with the tax-only serologic profile from the Bronx-Lebanon Hospital, and also from the Israeli hemophiliac. HTLV-II analysis was also included in the PCR study because the samples from the South Bronx and from Israel may be infected with this virus, although it is apparently not present in Japan. (The tax regions from HTLV-I and -II are highly homologous,³¹ therefore an HTLV-I-derived tax protein would be likely to serve as an antigen for antibodies provoked by an HTLV-II infection.³²) Four confirmed HTLV-II-positive blood samples (provided by Serologicals, Pensacola, FL) were used as positive controls.

All 19 of the healthy seropositive ATL family members and Japanese blood donors we tested were PCR-positive using the most sensitive primers (SK43 and SK44); most of the samples tested were positive using the other HTLV-I primers (Table 1). None of the four individuals that had antibodies to tax, and not to structural proteins, had detectable amounts of HTLV-I or -II DNA in their PBMCs by PCR amplification (Table 1). Not all primers had equivalent sensitivity. Some required only 2 to 3 molecules in a background of 2 μg of DNA to be initially present for amplification, others required 100 molecules for detection of the PCR product by dot blot. A serial dilution of HTLV-I-infected HUT-102 cells was included in each experiment to determine the sensitivity. The sensitivity listed in Table 1 refers to the particular reaction sets in which the DNA from the people with antibodies to HTLV-I tax-only were tested. The specificity of the signal on the dot blot was verified by multiple negative controls included in each reaction set (see Materials and Methods).

Analysis of baboon samples. A baboon population that was naturally infected with simian T-cell leukemia virus (STLV) was screened to determine if an analogous tax-antibody–only situation occurs in baboons; STLV shares a 90% to 95% DNA sequence homology with HTLV-I,³² and is serologically cross-reactive.³³-³⁵ Of 118 baboons tested, 24 (20.3%) had antibodies that reacted with structural proteins when tested by HUT-102 WB; of the 83 baboon sera that were structural protein antibody-negative, four (4.8%) were positive when screened by tax WB. The serostatus of the four that reacted tax-only–positive, along with positive and negative controls, was confirmed by HUT-102 RIP and
HTLV-1 tax ANTIBODY ONLY SEROLOGIC PROFILE

Table 1. PCR Sensitivity for Detection of HTLV Genome in PBMCs of HTLV Seropositives

<table>
<thead>
<tr>
<th>Primers</th>
<th>Genomic Location</th>
<th>No. of Molecules Detected</th>
<th>PCR⁺/HTLV-I⁺</th>
<th>PCR⁺/HTLV-II⁺</th>
<th>PCR⁺/tax⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK43-SK44²⁶</td>
<td>HTLV-I or II tax</td>
<td>2</td>
<td>19/19</td>
<td>4/4</td>
<td>0/4</td>
</tr>
<tr>
<td>RM3-RM4</td>
<td>HTLV-I, LTR</td>
<td>20</td>
<td>18/19</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>RM1-RM2alt</td>
<td>HTLV-I, env</td>
<td>20</td>
<td>0/5</td>
<td>4/4</td>
<td>0/4</td>
</tr>
<tr>
<td>BKg1-BKg2</td>
<td>HTLV-I, gag</td>
<td>100</td>
<td>7/7</td>
<td>NT</td>
<td>0/4</td>
</tr>
<tr>
<td>RS79-RS80²⁶</td>
<td>Human genome, β-globin</td>
<td>NT</td>
<td>19/19</td>
<td>4/4</td>
<td>4/4</td>
</tr>
</tbody>
</table>

The number of molecules detected refers to the least number of molecules required for detection in the reaction sets in which the DNA from people with tax-only were tested, as determined by serial dilutions using a known number of HTLV molecules (see Materials and Methods). PCR⁺/HTLV-I⁺ refers to the number of PCR-positive samples over the number of healthy Japanese HTLV-I structural antibody-positive people tested by PCR. PCR⁺/HTLV-II⁺ refers to the number of PCR-positive over the number of confirmed HTLV-II tested by PCR. And PCR⁺/tax⁺ refers to the number of PCR-positive over the number tested by PCR that had antibodies only against the tax protein, and not the structural proteins. All PCR primers were tested with 8 to 10 control DNAs from seronegative donors (the negative control DNA was co-purified with the samples described above). RM3/4 and SK43/44 were also tested with 31 seronegative ATL family members. All seronegative controls were negative using HTLV primers and positive using β-globin primers.

Abbreviation: NT, not tested.

Cambridge BioTech ELISA. A set of six baboons was bleb for PCR analysis, including two seronegative baboons, two seropositive baboons, and two baboons that had antibodies that reacted only with tax, and not structural proteins. The two structural antibody-seropositive baboons were PCR-positive, and the two seronegative baboons were PCR-negative. Of the two baboons that had the tax-only antibody reactivity, a single baboon was clearly infected by STLV using PCR analysis with four different primer pairs. This result shows that the tax-only serostatus can reflect an active STLV infection in peripheral blood cells. The other tax-only baboon was PCR-negative, as we found in the human samples.

DISCUSSION

Several lines of experimental evidence suggest that the HTLV anti-tax antibody response found in people with no detectable antibodies against HTLV structural proteins reflects contact with the HTLV virus. First, the anti-tax-only phenotype is associated with a high risk of HTLV infection, as shown by seroanalysis of the ATL family members. In the Miyazaki prefecture of Japan (an HTLV-I-endemic area), of 82 family members of ATL patients that lacked antibodies to virus structural antigens, seven (8.5%) had a specific antibody reaction exclusively to the HTLV-regulatory protein tax. In a previous study, 40 serum samples from a population with a lower prevalence of HTLV seropositivity (1% to 2%) were anti-tax antibody negative. Second, the response is specific: both the bacterial 50-Kd tax fusion protein used as antigen in the r-tax WB and the natural 42-Kd tax protein produced by HTLV-infected cell lines in RIP analysis serve as antigen for the people who have an anti-tax antibody response. The blocking assay also clearly shows that the tax-only positive serum specifically recognizes the epitope of the tax protein. Third, this type of antibody response was found in several populations at risk for HTLV infection, as well as a baboon colony that was naturally at risk for STLV infection. Fourth, the example of the seroconverter who developed a general antibody reaction also gives some evidence that the tax-only antibody response is specific for HTLV-I. We also searched protein and DNA data banks in an attempt to detect protein sequence homology between tax and known protein sequences. No significant homology was observed, which decreases, but does not eliminate, the likelihood that the anti-tax response was due to a cross-reaction with an unrelated antigen.

While the above arguments suggest that the individuals having the HTLV tax-only antibody response have been exposed to the virus, the PCR-negative PBMCs from these people leave some questions unanswered: do they have an active infection; should they be diagnosed as positive; and are they a risk to the blood supply? These people, if infected at all, may generally be infected at very low levels. Also, rates of HTLV tax-only antibody reactors in the blood supply of low incidence regions such as the United States remain unknown and will require a large survey to determine.

There are several possible explanations for the positive tax-only antibody response and the PCR-negative result. The virus may be present in the PB below our threshold of detection with PCR. If this is the case, the virus may be present in people with the tax-only response at lower levels than in people with antibodies to structural proteins because virtually all PBMC samples tested from healthy HTLV (both I and II) carriers with anti-HTLV structural antibodies tested PCR-positive using at least two primer pairs. Compatible with this explanation is the possibility that the virus may be sequestered away from the PB in such individuals. Further studies with other tissues (eg, bone marrow, lymph node) of the STLV-infected baboon population could address this question. A second possibility is that, in the case of people with a tax-only antibody response, the virus was eliminated by the immune response of the infected individual and the anti-tax antibody lingers beyond the time it took for the structural antibody response to decline. Yet, the STLV-infected PCR-positive baboon with the tax-only serologic pattern indicates that, at least in this system, the tax-only serology can be an indicator of a definite infection.

An alternative explanation is that an anti-tax response is mounted before a structural antibody response, possibly

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when the virus is still at sufficiently low levels to be below the threshold of detection by PCR (in our case, 2 molecules in a background of DNA from 280,000 cells). The two cases from which we have serial sera support this hypothesis. The Israeli hemophiliac converted from an HTLV-1 seronegative status to an HTLV tax-only seropositive. The woman from Miyazaki was tax-only seropositive in the earliest available serum sample, but converted to gp61-positive in subsequent samples. The easiest explanation for this case would appear to be that the virus was temporarily sequestered or maintained in a replication inactive form in which only the tax antigen was released in sufficient amounts to stimulate antibodies.

The hypotheses presented above are not mutually exclusive. In fact, the tax-only phenotype may have different underlying biologic reasons in different individuals. It is difficult at this juncture to say if these individuals pose a threat to the blood supply, or how they should be diagnosed. Certainly it would be useful to incorporate testing for HTLV tax-only serologic profiles in future studies of the antibody response to HTLV. Prospective studies may show the nature of the HTLV-immune response in these individuals.

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A Okayama, B Korber, YM Chen, J Allan, TH Lee, S Shioiri, N Tachibana, K Tsuda, N Mueller and MF McLane