Demonstration of Antibodies to Human T-Cell Lymphotropic Virus-I tax in Patients With the Cutaneous T-Cell Lymphoma, Mycosis Fungoides, Who Are Seronegative for Antibodies to the Structural Proteins of the Virus

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Although most patients with the cutaneous T-cell lymphoma, mycosis fungoides (MF), are seronegative for human T-cell lymphotropic virus-I or -II (HTLV-I/II) when tested by assays that measure only antibodies to the viral structural proteins, the majority of such patients harbor HTLV-I-related pol and tax proviral sequences that encode proteins not included in routinely used serologic tests. Tax mRNA has also been detected in their peripheral blood mononuclear cells (PBMC). Therefore, it seemed possible that these patients have antibodies to the tax protein. To investigate this, enzyme-linked immunosorbent assays (ELISAs) and Western blot assays were set up, using as antigens the full-length HTLV-I tax cloned from the prototypic HTLV-I-infected cell line, C91PL, and from PBMC of a MF patient, as well as a synthetic peptide made to the carboxy-terminal 20 amino acids of tax-I. Of 60 MF patients whose PBMC were shown to be positive for tax proviral DNA and mRNA, 50 (83%) were shown to have tax antibodies. The antigen derived from the MF patient was most useful in detecting such antibodies. These results demonstrate the need for including other HTLV-related antigens in addition to gag and env in serologic tests used to identify HTLV-infected individuals. The findings underscore the fact that individuals considered seronegative on the basis of currently used tests can be infected with HTLV.

WHEN STANDARD serologic tests are used, the majority of patients with the cutaneous T-cell lymphoma, mycosis fungoides (MF), are negative for antibodies to human T-cell lymphotropic virus (HTLV-I/II). Yet we have shown recently that the peripheral blood mononuclear cells (PBMC) and skin biopsies of almost all such patients harbor HTLV-I proviral sequences. In the course of further analysis, it was noted that specimens from most MF patients lack sequences that encode the viral structural proteins, gag and env, the antigens used almost exclusively in standard serologic tests. Antibodies generated against proteins encoded by the pol and pX regions of the HTLV genome, the very sequences that are demonstrable in PBMC of MF patients, are usually not assayed in such tests. Although it is considered possible that MF patients are to some degree immunocompromised, it is also conceivable that such patients actually make antibodies to proteins encoded by regions of the viral genome now known to be present in their cells. The latter possibility was strengthened by preliminary data showing that patients whose cells harbored pX-region proviral DNA sequences related to the transforming gene of the virus, tax, also expressed tax mRNA. To test the hypothesis that MF patients indeed make antibodies to HTLV gene products produced by their PBMC, the sera of 60 patients whose PBMC were shown to have tax proviral sequences and tax mRNA were tested by enzyme-linked immunosorbent assays (ELISAs) and Western blot assays using recombinant tax-I and a synthetic peptide to the 20 carboxy-terminal amino acids of tax-I as antigens. Of 60 patients tested, 50 (83%) proved to have demonstrable tax antibodies. The range of immunologic reaction and its possible significance is the subject of this report.

MATERIALS AND METHODS

Patients and Specimens

Sixty biopsy-proven patients with the cutaneous T-cell lymphoma, MF, or its leukemic variant, Sézary syndrome, who have been demonstrated to possess proviral DNA sequences related to HTLV-I tax in their PBMC were included in this study to determine whether they also have antibodies to the tax protein. The study subjects included 43 whose clinical and laboratory features were reported in detail previously and 17 who have been enrolled in the study subsequently.

Paired sera and PBMC were prepared from whole blood from each of the patients by Ficoll (Pharmacia, Piscataway, NJ)-Hypaque (Sanofi-Winthrop, New York, NY) gradient centrifugation as routinely performed in this laboratory.

Reverse Transcriptase–Polymerase Chain Reaction/ Southern Analysis

Reverse transcriptase–polymerase chain reaction (RT-PCR) was performed essentially as described by others, modified as follows. Total cellular RNA was isolated from lysates of approximately 1 to 2 × 10^6 PBMC using Trizol reagent (GIBCO-BRL, Gaithersburg, MD) in the presence of RNasin (20 U per reaction), followed by extraction with chloroform and precipitation with isopropanol. Reconstituted samples were treated with DNase I (GIBCO-BRL; 0.5 U per reaction) for 30 minutes at 37°C, followed by incubation for 10 minutes at 65°C in the presence of 20 mmol/L EDTA, pH 8.0, to inactivate the nuclease. Just before reverse transcription, samples were denatured at 90°C for 5 minutes. First-strand cDNA synthesis was performed using M-MLV RT (GIBCO-BRL; 200 U per reaction), 50 pmol HTLV tax/pX primer SK43, and the buffer conditions used in standard DNA PCRs. dNTP concentrations used during reverse transcription were fivefold higher than those used for PCR, in a final reaction volume of 20 μL per sample. Positive and negative controls included lysates of the prototypic HTLV-I and -II cell lines,
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C91PL, and MoT, respectively, and PBMC isolated from HTLV-I/II tax PCR-negative volunteers. Reverse transcription was performed at 37°C for 60 minutes, followed by boiling for 10 minutes to inactivate the RT. Subsequently, PCR amplification was performed in the same tubes upon addition of tax/pX primer SK44 (50 pmol per reaction). Tag polymerase (Perkin Elmer, Foster City, CA; 2.5 U per reaction), and sufficient 1× PCR reaction buffer to bring the final volume to 100 μL. No further additions of SK43 or dNTPs were required. The amplification times and temperatures used for PCR and details pertaining to Southern blot analysis using digoxigenin-tailed probes have been described previously.2

ELISA and Western Blot Assays

Preparation of Antigens

Recombinant full-length HTLV tax-I protein. Provilal DNA sequences spanning the entire tax open reading frame in the pX region of HTLV-I were amplified by PCR from the prototypic HTLV-I cell line, C91PL, and from PBMC of MF patient no. 1. The 5' sense and 3' antisense primers used to amplify these tax sequences included 5'-GGCGGATCCTGCCCCATTCCACAGG-3' and 5'-GGGGGATCCATGGCCCAC'TCCCAGC-3' (genomic and 3' antisense primers used to amplify these tax sequences are underlined). Additional sequences included coding sequences for BamHI and EcoRI restriction enzyme cleavage sites added to the sense and antisense primer sequences, respectively, to facilitate cloning of the tax sequences in frame into the GST fusion protein expression vector, pGEX-2T. The recombinant GST–tax-I fusion proteins were expressed in Escherichia coli BL21 cells and then isolated from such cultures by chromatography using glutathione linked to Sepharose-4B (Pharmacia, Piscataway, NJ). For some experiments, purified tax protein free of glutathione-S-transferase (GST) was prepared by thiombin cleavage, removal of GST fragments by selective binding to glutathione-Sepharose, and removal of thiombin by chromatography through columns of benzamidine-Sephrose-6B. Reagents and protocols for these procedures were obtained from Pharmacia.

Synthetic carboxy-terminal tax-I peptide. A peptide corresponding to the 20 carboxy-terminal amino acids of HTLV tax-I (QISPGG-LEPPSEKHFRETEV), known to be HTLV type-specific and immunodominant in humans, was synthesized at our institution as described previously.2

Assays

HTLV tax antibody ELISA. HTLV tax antibody ELISA was performed essentially as described previously,1 using reagents obtained from Pierce Chemical Co (Rockford, IL). Microtiter wells coated with 2 μg per well of the recombinant full-length tax-I proteins from the HTLV-I infected cell line, C91PL, or from PBMC of MF patient no. 1 or the carboxy-terminal tax-I synthetic peptide. Initial screenings of sera with the recombinant proteins were performed using the GST–tax-I fusion proteins as antigens. Samples that proved positive in these assays were retested with purified tax proteins free of the GST sequences. Serum dilutions ranged from 1:10 to 1:320. Reactions were developed using goat anti-human IgA + IgG + IgM (H + L-chain) antibodies, conjugated with horseradish peroxidase (HRP) in the presence of the HRP substrate, ABTS (Pierce Chemical). Tests were read at 410 nm, and samples registering more than 3 standard deviations above the mean obtained from measuring responses in sera from 25 HTLV PCR tax sequence–negative volunteers were considered positive. Serum from a patient with tropical spastic paraparesis (TSP)-HTLV-I–associated myelopathy (HAM) served as the tax antibody–positive control.

HTLV tax antibody Western blot assays. Purified recombinant tax-I protein was resolved by preparative sodium dodecyl sulfate–polyacrylamide gel electrophoresis through 8% polyacrylamide minigels, followed by electrophoretic transfer to nitrocellulose.22 Blots were blocked for 1 hour at room temperature by incubation in 150 mM NaCl, 100 mM Tris-hydrochloride, pH 7.5, and 2% Blocking Reagent (Boehringer-Mannheim, Indianapolis, IN), followed by extensive washing in TBS (20 mMOL Tris-hydrochloride, pH 7.5, 500 μM NaCl). Membranes were then cut into strips or processed using the Bio-Rad (Hercules, CA) mini-Protein II multiscreeen apparatus and then incubated for 1 hour at room temperature, with sera from MF patients or controls, diluted 1:10 in blocking buffer. After three 5-minute washes in 0.05% Tween 20 in TBS, the strips were further incubated for 2 hours at room temperature in secondary antibody (goat anti-human IgA + IgG + IgM, H + L chains, conjugated with alkaline phosphatase; Pierce Chemical Co). After three washes in Tween 20/TBS, the strips or blots were rinsed in 100 mMOL Tris-hydrochloride, pH 9.5, 100 mMOL NaCl, and 50 mMOL MgCl2, and finally incubated with the alkaline phosphatase substrates, 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate. Known HTLV-positive and -negative sera were included in each assay, along with colored molecular-weight markers (Amersham Corp, Arlington Heights, IL), to aid in identifying MF patients who were positive for tax antibodies. The sera from 25 healthy, HTLV tax PCR negative volunteers used to set up the ELISA were all negative by Western blot assay. At least one known HTLV PCR-negative serum sample was included in each of the assays in which sera from MF patients were tested. The GST-tax and thrombin-cleaved tax proteins were identified on Western blots using a polyclonal rabbit antisemur to recombinant full-length tax originally cloned into and expressed by a baculovirus expression vector, obtained from the National Institutes of Health AIDS Research and Reference Reagent Program, and goat anti–rabbit IgG conjugated with alkaline phosphatase (Pierce Chemical) as described earlier.

PCR/Southern Blot Analysis

The procedures used to prepare whole-cell lysates and to perform PCR/Southern blot analysis to detect HTLV tax proviral DNA sequences in PBMC from MF patients, have been described in detail previously.2 The HTLV tax primers included SK43/44, to amplify a 159-bp region between HTLV-I genome sequences 7358 and 7516, and 5' sense and 3' antisense primer sequences 5'–GAAATACGACATCCACCC-3' and 5'–TCAGACCTGCTTGTTCGGC-3', respectively, to amplify the 132 carboxy-terminal bp of the tax open reading frame in the pX region of HTLV-I, map locations 7652 to 7784. The sequences amplified at the carboxy terminus of the tax-I open reading frame encompass the coding sequences for the tax-I synthetic peptide used in tax antibody ELISA tests. Positive and negative controls for PCR included in each assay consisted of the prototypic HTLV-I and -II cell lines and PBMC isolated from HTLV-I/II tax PCR-negative volunteers. Primers specific for amplification of β-globin (PC04 and GH20)25 were used to verify HTLV-negative PCR results. β-Globin-related amplification products were visualized after electrophoresis through agarose gels containing ethidium bromide. Sufficient amplification products were detected such that Southern blot analysis was not required.

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Results

Detection of tax mRNA in PBMC from patients with MF. Using SK43 as the primer in RT assays, SK43/44 for PCR,
and digoxigenin-tailed SK45 in Southern analysis, tax-related mRNA was readily detected in PBMC of all 60 patients tested. A representative Southern blot of RT-PCR/Southern analysis experiment showing detectable tax mRNA in lysates of PBMC from nine MF patients is shown in Fig 1.

**Detection of HTLV-I tax antibodies in sera from patients with MF.** Fifty of 60 patients who have been shown to have tax sequences and tax mRNA proved also to have antibodies to tax when r’ MF tax-I was used as the antigen (Table 1). Representative Western blot assay data are also shown in Fig 2 for 10 different patients. Sera from three patients reacted with both wild-type (WT) and MF patient-derived tax antigens (lanes to 3 to 5), whereas sera from five others reacted only with the patient-derived antigen (lanes 6 to 10) and two were nonreactive with both antigens (lanes 11 and 12). Sera from three different healthy, HTLV PCR-negative volunteers were tested in lanes 13 to 15. Interestingly, the sera of 19 patients that reacted with r’ MF tax did not react with r’ WT tax, the antigen derived from the HTLV-I-infected cell line, C91PL. Moreover, only 17 of 50 positive sera reacted with the synthetic carboxy-terminal peptide of tax-I, which by some observers has been considered an immunodominant region of the protein.19,20

The specimens of 17 patients failed to react with either the recombinant WT tax antigen or the synthetic peptide—even when their sera were diluted minimally (1:10). However, using a recombinant antigen prepared from PBMC of an MF patient, sera from 17 of these latter individuals were reactive. None of the patients had detectable tax antibodies in ELISA tests at serum dilutions greater than 1:160. All of the positive ELISA results obtained using the recombinant full-length tax antigens were confirmed by Western blot assays, using purified (thrombin-cleaved, GST sequence-deleted) tax antigens. To date, there remain 10 MF patients of 60 tested in whom tax antibodies have not been demonstrated, although all have tax proviral sequences and mRNA demonstrable in their PBMC.

It should be emphasized that only three of 60 patients in this study (patients no. 1, 9, and 18) were seropositive for HTLV-I/II antigens when the sera were assayed using routine tests that contain only the viral structural proteins as antigens. Based on the findings presented here, the prevalence of antibodies to HTLV in MF patients is actually significantly higher (50 of 60 or three of 60) when more relevant antigens are used in the tests.

**Detection of carboxy-terminal tax proviral DNA sequences by PCR/Southern analysis.** The reason the patients’ sera were tested for antibodies directed against the carboxy-terminal portion of tax was that this sequence had been considered immunodominant by some investigators.19,20 Unexpectedly, the sera of only 17 patients who reacted with the r’ MF tax reacted with the tax peptide. PCR/Southern analysis showed that the cells of eight of 33 negative patients lacked the proviral DNA sequences necessary to encode this region of the tax molecule, while they harbored proviral DNA sequences, mRNA, and antibodies to other regions of the tax open reading frame and its gene products, respectively.

**DISCUSSION**

The PBMC of 60 patients with MF shown to harbor HTLV tax proviral sequences have now also been shown to express tax mRNA (Fig 1). In addition, 50 (83%) of these 60 patients had detectable tax antibodies (Table 1), even though only three (patients no. 1, 9, and 18) had antibodies to gag and/
or env as measured by standard commercially available tests that use minimal serum dilutions of 1:100 (and unpublished observations in this laboratory). Since PBMC from most of these patients also lacked gag and env proviral sequences, their seronegative status in standard serologic tests is not surprising. Defective forms of HTLV-I have been observed in PBMC of patients with adult T-cell leukemia/lymphoma (ATL), tropical spastic paraparesis (TSP), and HTLV-I–associated myelopathy (HAM).\textsuperscript{25–27} When characterized in detail, such defective viral forms preferentially retained tax/pX-region sequences. It is not known whether defective HTLVs in such patients are transcriptionally active, or whether ATL/TSP-HAM patients harboring defective HTLV are able to mount immune responses specific to viral gene products relevant to the defectives. However, such information would be difficult to obtain, because most ATL and TSP-HAM patients also harbor intact, infectious viruses and have antibodies to the viral structural proteins.

In patients with ATL and TSP-HAM, as well as in healthy carriers of HTLV-I from regions of the world endemic for the virus, the antibody response to the tax protein has variously been correlated with viral load, ie, the greater the virus concentration in peripheral blood cells, the higher the antibody titer to tax and to the viral structural proteins.\textsuperscript{28,29} This, in turn, was correlated with the ease of transmission.\textsuperscript{30–32}

In three studies, ATL patients tended to have significantly lower levels of antibodies to the tax protein than found in patients with TSP-HAM or healthy carriers.\textsuperscript{20,33,34} Recently, Furukawa et al\textsuperscript{55} have shown that PBMC from ATL patients express lower levels of tax mRNA than cells from patients with TSP-HAM or cells obtained from carriers of the virus. Decreased antibody levels in ATL seem consistent with the high incidence of severe immunodeficiency observed in these patients.\textsuperscript{36}

In contrast, immunosuppression in MF patients has not been noteworthy. That such patients may to some extent be immunocompromised is suggested by the relatively low levels of tax antibodies detected in 50 of our subjects and the failure to detect tax antibodies in the remaining 10. PBMC from the latter 10 patients nevertheless had tax proviral DNA and mRNA. Since in endemic areas the virus is known to be transmitted from mother to child,\textsuperscript{31,32,37} it is possible that antibody-negative individuals who harbor defective viruses were made tolerant early in life.

The necessity for sequence amplification and Southern analysis to demonstrate both proviral DNA and mRNA in PBMC from MF patients is indicative of the low levels of defective HTLV in such patients.

Sera from 17 MF patients in this study reacted with the synthetic peptide prepared to the carboxy-terminal 20 amino acids of tax-I. This sequence is type-specific for HTLV-I,\textsuperscript{17,19,20} The HTLV-IIa tax protein is missing this region, whereas the carboxy-terminal amino acid sequence for type IIb tax is totally distinct.\textsuperscript{38–40} These results further confirm our published data based on PCR/Southern analysis that most MF patients harbor HTLV-I rather than HTLV-II.\textsuperscript{2} To date, only one MF patient among our study subjects has been found to be infected with HTLV-II.\textsuperscript{41} As for the 33 patients who were found to be seropositive for antibodies directed to r’ tax but lacked detectable antibodies to the carboxy terminus of tax-I, it is theoretically possible that some or all of the 33 harbored HTLV-II rather than HTLV-I. However, 24 of 33 have been shown by PCR/Southern analysis to harbor pol-I and not pol-II, in addition to tax (and unpublished results). Moreover, tax proviral sequences amplified from PBMC of patients no. 1, 4, and 22 were sequenced and found to be more closely related to tax of HTLV-I than of HTLV-II in the 159-bp region of tax analyzed.\textsuperscript{2} The nine
patients who were not shown to harbor pol-I sequences appear to lack HTLV pol sequences altogether, since pol-II–specific sequences also were not detected. Of 33 patients who did not produce antibodies to the tax carboxy-terminus, eight also lacked proviral coding sequences for this region of the tax molecule. These results support our previous observations indicating that most MF patients harbor a defective form of HTLV-1 than to HTLV-II.

The greater reactivities of sera from MF patients against the MF patient–generated r1 tax antigen versus the antigen prepared from the prototypic HTLV-I–infected cell line may well reflect mutated viral sequences that may have arisen during more than 12 years of continuous culture of the C91PL cell line.15

Although several genetic and functional studies have been undertaken to characterize the intact tax protein, more information has been gleaned about the ability of specific regions of the tax molecule to transactivate a variety of viral and cellular genes than has been revealed about the role(s) of tax-specific epitopes in transformation or leukemogenesis.42-46 The tax-I protein has been shown to be capable of interacting with cellular transcription factors of at least three distinct signal transduction pathways (NFKB/Rel and CREB/ATF,44-46 and more recently, the JAK/STAT7 pathways), ultimately to turn on a number of cellular growth-promoting genes, including oncogenes. Through analysis of mutations in tax-I that reciprocally block tax-directed actions through the NFKB and CREB pathways, Smith and Greene46 have found evidence that tax-mediated transformation of rat fibroblasts is likely to involve the CREB/ATF pathway. The pathway(s) important in transformation of lymphoid cells in vivo, and any role tax may play in this process, have yet to be fully defined. However, it has recently been shown that the NFKB pathway may not be obligatory for transformation of T lymphocytes by HTLV-I tax.48

This study clearly demonstrates the need to include antigens in addition to those related to the viral structural proteins in the diagnosis of HTLV infections by means of serologic tests. The results also show the utility of additional types of analyses (PCR/Southern and RT-PCR/Southern) to aid in the detection of infection with HTLVs. The latter is of particular importance, since the incidence of infection by HTLV-I and -II is on the increase worldwide, particularly among inner-city populations.49 Such combined analyses may be essential for determining the true prevalence of HTLV infection in the general population and for investigating whether defective HTLVs are transmitted.

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