

Quantitative Proviral DNA and Antibody Levels in the Natural History of HTLV-I Infection

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The pathogenesis of human T-cell lymphotropic virus type I (HTLV-I) in adult T-cell leukemia/lymphoma (ATL) and HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP) is poorly understood. We prospectively followed up and evaluated the virologic correlates of infection in transfusion recipients after seroconversion, in asymptomatic carriers, and in ATL and HAM/TSP patients. Proviral DNA levels (copies/10⁵ lymphocytes) were determined by real-time automated polymerase chain reaction and antibody titers by end-point dilution by use of an HTLV-I enzyme-linked immunoassay. In early infection, proviral load was initially elevated (median, 212 copies/10⁵ lymphocytes at time 1) and later decreased (median, 99 copies at time 2, and 27 copies at time 3). Corresponding antibody titers were low at time 1 (1 : 2154), had significantly increased by time 2 (1 : 12312), and were stable by time 3 (1 : 4694). These viral markers were significantly lower in asymptomatic carriers than in HAM/TSP or ATL patients. Therefore, proviral load and antibody titers may be useful as predictive markers of disease among carriers.

Human T-cell lymphotropic virus type I (HTLV-I) is the causative agent of adult T-cell leukemia/lymphoma (ATL) and the chronic neurodegenerative disorder, HTLV-I associated myelopathy, also known as tropical spastic paraparesis (HAM/TSP). These diseases cluster in areas endemic for HTLV-I: southern Japan, the Caribbean basin, parts of South America and Africa, and also in Europe and North America, areas where it is not endemic but with significant numbers of immigrants from endemic geographic locales. An increasing spectrum of HTLV-I related diseases also have been reported, including infective dermatitis of children [1], uveitis [2], and Sjögren syndrome [3]. However, the most common manifestation, affecting millions worldwide [4], is the asymptomatic, HTLV-I carrier state; carriers harbor persistent viral antibodies throughout their lives. The lifetime cumulative risk of carriers developing

ATL or HAM/TSP is 1%–5% [5, 6], and the annualized incidence of ATL and HAM/TSP in major endemic areas is 16 and 22 per 100,000 HTLV-I carriers, respectively [6, 7]. Excess morbidity caused by other illnesses among HTLV-I carriers has been reported in populations in which it is endemic [8, 9]. It has been postulated that this additional disease burden among carriers increases the lifetime risk of adverse clinical outcomes to about 10% [10].

Biomarkers to identify carriers at high risk for disease are not clinically available, primarily because critical virologic and immunologic events in the natural history of HTLV-I infection and disease pathogenesis are not well understood. Several investigators have found that proviral load may provide some insight on the transmission of the virus [11] and associated diseases [12, 13]. However, none have prospectively examined the patterns of proviral load in relation to HTLV-I antibody titers in early infection after HTLV-I seroconversion, which would be crucial information for determining the predictive value of these virologic markers. In this study, we provide a composite picture of the evolution of proviral load and antibody titers after recent HTLV-I infection, and of the carrier state and its potential relationship to disease outcome.

Materials and Methods

Study population. A prospective cohort study of transfusion transmission in Jamaica was established in May 1987. The study was designed to evaluate factors important to the transmission of

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HTLV-I and served as a basis for establishing policy regarding blood donor screening. The details of the study design have been reported elsewhere [14]. All enrollment was completed prior to availability of licensed HTLV-I screening assays in September 1988. Ongoing follow-up of the transfusion recipient cohort initially included monthly visits for 6 months, every 2-month visits for 6 months, and subsequent annual visits with blood samples obtained and physical examinations performed to assess the presence of HTLV-I associated outcomes.

In the original cohort, 24 transfusion recipients were identified as HTLV-I seroconverters with a median time to seroconversion of 51 days. For the current evaluation, serial serum and peripheral blood mononuclear cell specimens, at 3 time points after transfusion (between 1 and 3, 7 and 14, and >14 months [range, 56 days–5 years]), were examined from 10 representative transfusion recipients of HTLV-I positive blood who had several years of follow-up after HTLV-I seroconversion. In addition, to correlate virologic findings with HTLV-I associated diseases, specimens from HTLV-I seropositive patients who had clinical features typical of HAM/TSP infection ($n = 27$), ATL infection ($n = 30$), and asymptomatic adult carriers ($n = 50$) were selected from epidemiologic studies in the same geographic locale. Time and route of infection were not known for the carriers or patients.

Laboratory methods. HTLV-I seroconversion had been previously established in serum samples from the transfusion recipients by whole virus enzyme-linked immunoassay (EIA) (Dupont, Wilmington, DE) and confirmed by Western blot (Biotech, Rockville, MD). HTLV-I was distinguished from HTLV-II by Western blot or polymerase chain reaction (PCR). Antibody titers were assayed by the end-point dilution method by use of an EIA (Genetic Systems, Seattle, WA or Cambridge-Biotech, Rockville, MD) at 4-fold dilutions. Serum and peripheral blood lymphocyte samples had been stored at -70°C until used.

Quantitative proviral DNA levels were detected by a real-time automated PCR method. For each test sample, 10 μL of DNA (300 ng) was amplified for 45 cycles with AmpliTaq Gold polymerase by use of an ABI PRISM Sequence Detection System and TaqMan PCR Reagent [P/N N808-0230] (PE Applied Biosystems, Foster City, CA) in a 96-well format. The HTLV-I/II primers used in this study were from highly conserved sequences (GenBank National Center for Biotechnology Information, Bethesda, MD) from the *tax* gene and are designated as HTV-F5 (7358-7378) and HTV-R4 (7518-7499). Triplicate reactions were performed and unknown copy numbers were calculated by interpolation from the plasmid control regression curve and reported as copy equivalents per 10^5 lymphocytes. The assay reliably detects at least 3 copies/ 10^5 lymphocytes. Samples with undetectable virus were scored as 1 copy/ 10^5 lymphocytes for statistical calculations.

Statistical analysis. To identify virologic correlates of early HTLV-I infection among transfusion recipients and the disease groups, proviral load and antibody titer levels were treated as continuous variables. Median levels of proviral load and antibody titers were presented with associated 25th percentile and 75th percentile (interquartile range [IQR]) levels as descriptive summary statistics. Because of the nonnormal distribution of values among the groups even after \log_{10} transformation, nonparametric statistical methods were used to assess trends over time and make comparisons between groups. Among transfusion recipients, a simplifying as-

sumption was made that changes of proviral load and antibody titers were linear between visits. Differences of levels over time were assessed by 2 methods for related samples: Friedman's 2-way analysis of variance for testing differences on levels between time points [15, 16] and Page's test for ordered alternatives to test for an ordered trend over time [17]. Correlations between proviral load and antibody titer levels were examined by means of Spearman's rank order statistic.

To compare proviral load and antibody titers among asymptomatic HTLV-I carriers, ATL patients, and HAM/TSP patients, we treated all values as continuous variables. Each value was \log_{10} transformed and the geometric means as well as 95% confidence intervals (CIs) were calculated to make comparisons between groups. Separate logistic regression models were used for comparisons of proviral load and antibody titer levels between the 2 disease groups and HTLV-I carriers, adjusted for age and sex as potential confounders. Mean ages between groups were compared with the Wilcoxon Rank Sum test (χ^2 approximation), and sex differences between groups with χ^2 or Fisher's exact test. All log-transformed results were ultimately reported as arithmetic values. All P values were 2-sided. Statistical Analysis Software System (SAS Institute, Cary, NC) was used for computing most statistics.

Results

HTLV-I virologic correlates in early infection. First we evaluated proviral load and antibody titers in the HTLV-I transfusion recipients who became infected. The clinical and demographic features of the 10 transfusion recipients are presented in table 1. There were 6 women and 4 men, median age 35 years (range, 18–66), who had various diagnoses and indications for transfusion. The most common indication was acute blood loss caused by trauma or complications in the peripartum period. Three patients had intrinsic disease of the hematologic or cardiovascular system, including aplastic anemia, myelofibrosis, and congestive heart failure.

Proviral load and antibody titers for the 3 time points after transfusion are presented in table 2. In the early period after transfusion, from 1–3 months (median, 68 days), proviral load was elevated: median 212 copies/ 10^5 lymphocytes (25%–75% interquartile range [IQR], 53–1502); by the later 2 time points it had decreased: at 7 and 14 months (median, 222 days), me-

Table 1. Clinical and demographic features of transfusion recipients.

Recipient	Age	Sex	Diagnosis
RE0193	36	Male	Aplastic anemia
RE0508	18	Male	Burns
RE0645	33	Male	Chest wound, hemothorax
RE1038	40	Male	Postoperative hemorrhage
RE1142	18	Female	Liver disorder of pregnancy, anemia
RE1659	57	Female	Heart failure, hypertension, ascites
RE3013 ^a	66	Female	Myelofibrosis, chronic anemia, ascariasis
RE5359	28	Female	Pregnancy, preeclampsia
RE6710	27	Female	Pregnancy, anemia
RE6873	39	Female	Pregnancy, anemia

^a RE3013-subsequently developed human T-cell lymphotropic virus type I-associated myelopathy.

Table 2. Proviral DNA and antibody levels after human T-cell lymphotropic virus type I seroconversion in transfusion recipients.

Time after transfusion, months	Median proviral DNA levels, copies/10 ⁵ lymphocytes	Median reciprocal antibody titers
1-3	212 (53, 1502) ^a	2154 (195, 5494)
7-14	99 (23, 333)	12,312 (8962, 22,044)
>14	27 (4, 340)	4694 (1200, 10,996)

^a 25th and 75th percentile values.

dian load was 99 copies/10⁵ lymphocytes (IQR, 23-333), and at >14 months (median time, 1162 days), median load was 27 copies/10⁵ lymphocytes (IQR, 4-340). A comparison of differences in proviral load between time points was not statistically significant ($P = .25$). However, mean proviral load was observed to decrease over time with a significant trend ($P < .05$).

Antibody titer was initially low in the 1-3 month period after transfusion (1 : 2154 [IQR, 1 : 195-1 : 5394]), but increased at 7-14 months (1 : 12,312 [IQR, 1 : 8962-1 : 22,044]) and remained high after >14 months (1 : 4694 [IQR, 1 : 1200-1 : 10,996]). A comparison of mean antibody titer levels between time points was significant ($P = .002$). The change in titer that was driving the overall association was the difference from time 1 to time 2 compared with the difference from time 2 to time 3 ($P = .006$). Antibody titer at time 1 was lower than titers at times 2 or 3, with a significant trend over time ($P < .001$).

The patterns of proviral load and antibody titer among individual recipients are shown in figure 1. The majority of recipients had an initial increase with subsequent decline in proviral load after 1 year. Only 20% had low initial levels and then stabilized at a higher proviral load. At least 50% had levels below 100 copies/10⁵ lymphocytes about 1 year after infection, and among these recipients nearly half had levels that were below the level of detection. A dynamic relationship was observed between proviral load and antibody titer over time: proviral loads at times 1 and 3 were significantly correlated with each other ($R = .74$; $P = .01$); proviral load at time 2 was significantly correlated with antibody titer at time 2 ($R = .82$; $P = .004$) and at time 3 ($R = .81$; $P = .005$). Antibody titer at times 2 and 3 were significantly correlated with each other ($R = .95$; $P = .0001$).

Virologic determinants of HTLV-I related disease progression. One transfusion recipient (RE3013, table 1 and figure 1) in the cohort, a 66-year-old woman, developed HAM/TSP during the period of observation. Neurologic symptoms developed ~3 years after transfusion. As shown in table 3, initial proviral load (1502 copies/10⁵ lymphocytes) measured 83 days after transfusion was at least 10-fold higher than the average proviral load determined for other recipients in the same time period. However, by 222 days her proviral load declined (to 333 copies/10⁵ lymphocytes) and by 2.7 years posttransfusion it reached a nadir (103 copies/10⁵ lymphocytes). In contrast, her antibody titers were initially low (1 : 93) and increased within the first year to a titer nearly 4-fold higher than the

median titer for the seroconverter cohort at the corresponding time point (1 : 45,822 vs 1 : 12,312). Her antibody titer (1 : 52,890) reached a maximum at 2.7 years, quite comparable to the median titer (1 : 69,199) of our prevalent HAM/TSP patients (described in next section). Proviral load subsequently increased to 2915 copies/10⁵ lymphocytes at a time when neurologic symptoms were advanced. Interestingly, her peak antibody titer was correlated with onset of neurologic symptoms and signs of HAM/TSP and with the nadir of her proviral load (103 copies/10⁵ lymphocytes). Throughout the period of observation, her antibody titer was elevated compared to the other carriers, except 1 (RE1659; figure 1). Her proviral load was elevated compared to the other carriers but only after development of advanced disease. However, a critical sample was unavailable within the first year after symptom onset. RE1659, a female recipient of similar age and duration of follow-up, had higher antibody titers and proviral load but did not develop disease prior to death, ~3.5 years after transfusion. This suggests that other factors may also be important in disease pathogenesis.

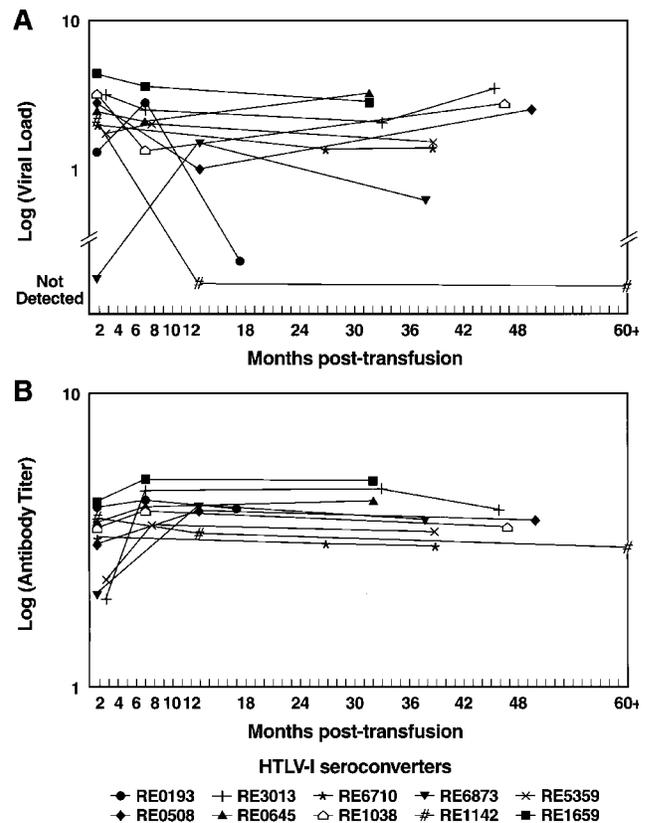


Figure 1. Patterns of human T-cell lymphotropic virus type I (HTLV-I) proviral DNA and antibody titer (as reciprocal) levels among transfusion recipients after seroconversion for 3 time points with median times of 68, 222, and 1162 days. *A*, proviral DNA levels as copies per 10⁵ lymphocytes; *B*, antibody titers.

Table 3. Case summary: transfusion-associated human T-cell lymphotropic virus type I-associated myelopathy (also known as tropical spastic paraparesis) in a 66-year-old woman.^a

Time	Clinical events	Proviral DNA level ^b	Reciprocal antibody titer
5–8/87	Transfusion	1502	93
12/87	None	333	45,822
1/90	3 months of weakness, paresthesias in lower limbs with moderately increased reflexes	103	52,890
8–11/90	50% power in lower limbs with Babinski sign, increased reflexes and tone	NA	NA
3/91	Urinary urgency, paraparesis	2915	10,676
10/91	Septicemia; death		

NOTE. NA, not available.

^a Admitting diagnosis: myelofibrosis since 3/87

^b Copies/10⁵ lymphocytes

Proviral DNA and antibody titers in the HTLV-I carrier state and disease. To further examine the relationship between proviral load, antibody titer, and established disease, we evaluated biospecimens from prevalent ATL patients, HAM/TSP patients, and asymptomatic carriers (figure 2). Our sample of ATL and HAM/TSP patients was representative of such patient groups in Jamaica. They had median ages of 43 years (range, 14–85) and 49 years (range, 28–69), respectively. HTLV-I carriers had a median age of 38 years (range, 20–77), which was significantly ($P = .03$) younger than the HAM/TSP patients. As expected, ATL patients were equally divided between males (53%) and females (47%), whereas HAM/TSP patients were predominately female (70%). HTLV-I carriers were predominately female (84%), and this distribution significantly differed from the ATL patient group ($P = .001$). Therefore, comparisons between groups were adjusted for age and sex.

The distribution of proviral load and antibody titer levels for prevalent HTLV-I carriers, and ATL and HAM/TSP patients are presented in figure 2. Proviral load had a broader range in values than did antibody titer levels. This was particularly true among HTLV-I carriers, which suggests that the variation might be caused by differences in disease susceptibility or duration of infection. Several carriers had proviral load below the level of detection, although corresponding antibody titer levels were present. Proviral load was significantly higher among ATL patients compared to HTLV-I carriers ($P = .0001$) and also compared to HAM/TSP patients ($P = .009$). Conversely, HAM/TSP patients had proviral load only slightly higher than HTLV-I carriers ($P = .06$).

In contrast, antibody titer levels were higher among HAM/TSP compared to HTLV-I carriers ($P = .0002$) and also were higher among ATL patients compared to HTLV-I carriers ($P = .002$). In contrast, antibody titer levels for ATL and HAM/TSP patients were similar ($P = .22$). Consequently, proviral load more clearly distinguished ATL from HAM/TSP patients.

Discussion

Proviral DNA levels represent a measure of integrated viral genome in host cells [18] and a surrogate marker of HTLV-I

viral replication [19]. HTLV-I antibody titer reflects the host's humoral immune response to infection, since production of antibodies is important to diminishing the infectivity and the number of infected cells [20]. Determination of the relationship of these virologic markers is important for understanding the pathogenesis of HTLV-I and related diseases. In our prospective analysis of the natural history of HTLV-I infection among seroconverters, we have delineated the virologic events in early infection. We have shown that early in the course of infection proviral load was high but was rapidly controlled in most cases. Within the first 90 days of infection, a narrow range of proviral load was observed, and we found that this was highly correlated with levels after 1 year of infection. This suggests that soon after infection a steady state was established under the influence of the host's immune response. Antibody titer was highly correlated with proviral load after the initial set point was established, which implies that the antibody response was driven by the early host-virus interaction under the influence of cell-mediated immunity.

The pattern of viral levels observed over time was consistent with acute viral infection followed by chronic persistent infection, a pattern similar to that observed for other retrovirus infections. An analogous response has been reported for primary human immunodeficiency virus type 1 (HIV-1) infection [21]: after an initial increase, a rapid decrease in HIV-1 RNA viral levels was observed in the first 120 days of acquisition. In the HIV-1 setting during the first 30 days after infection, there is wide variability between individuals. It was surprising that the range of HTLV-I proviral load values in our study was narrow, and no significant differences in proviral load were observed between individual transfusion recipients. This may have been caused by the presence of small numbers of infected cells, the highly cell-associated properties of HTLV-I, insensitivity of the quantitative methods at low viral levels, or similarity of subjects in our modest sample. We also may have missed peak viremia because our earliest samples were taken at a median of 68 days. Nonetheless, in this investigation, early infection with HTLV-I and the resultant chronic carrier state appears to be characterized by low viral copy numbers. Con-

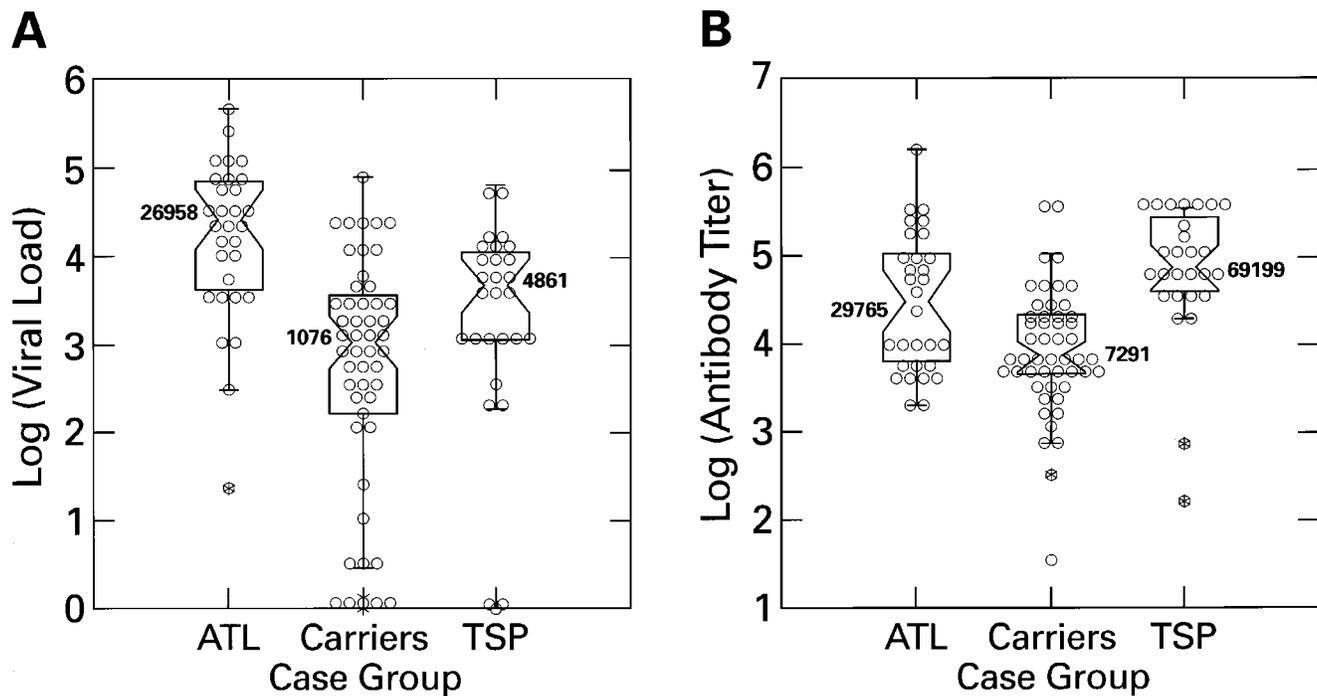


Figure 2. Scatterplots and boxplots of proviral DNA levels and antibody titer (as reciprocal) levels for adult T cell leukemia/lymphoma (ATL) ($n = 30$), human T-cell lymphotropic virus type I (HTLV-I) carriers ($n = 50$), and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) ($n = 27$) patients. Box plots are interpreted as follows: waist, median; diagonal lines, 95% confidence intervals about median; lower and upper horizontal lines, 25th and 75th percentiles of distribution; upper and lower bars, range of data excluding outliers; *, outliers; and \circ , far outliers. Medians are also shown as arithmetic values of log transformed results. *A*, proviral DNA levels as copies/ 10^5 lymphocytes; *B*, antibody titers.

sequently, proviral load and antibody titer levels may be useful as predictors of subsequent disease progression.

It is rare that the opportunity exists to prospectively evaluate disease progression in HTLV-I infection, because of the infrequent development of disease among carriers and the long latency between infection and disease onset. HAM/TSP has an onset of weeks to years after transfusion-acquired infection [22], whereas duration of disease latency after sexual acquisition of HTLV-I is not known. ATL has a disease latency of 10–40 years, with childhood acquisition of virus as most important for subsequent risk of disease [23]. In a recent report among prospectively evaluated asymptomatic carriers, several cases of ATL developed in a 10-year period of observation [24]. The authors estimated that carriers with the highest antibody titers had an ~70-fold risk of developing ATL relative to those with lowest titers. The study also showed that prediagnostic sera among the ATL cases revealed low or undetectable levels of antibody to the HTLV-I regulatory protein, Tax (anti-Tax), for ≤ 10 years prior to diagnosis. Therefore, the combination of these viral markers may predict carriers at risk for ATL.

In the current study, we observed the development of HAM/TSP in a single transfusion recipient after HTLV-I seroconversion. This patient had a hematologic disorder and parasitic

infection that may have contributed to a heightened immune response after infection with elevated antibody titers preceding development of HAM/TSP. In addition, the patient was transfused with multiple HTLV-I–positive blood components. Despite these factors, the patient demonstrated initial immunologic control and a decrease in proviral load in the first year of infection, with proviral load reaching a nadir near the time of symptom onset, 2.7 years after transfusion. This patient’s proviral load was increased in association with advanced disease, when evaluated ~4 years after seroconversion. In an earlier report, we also demonstrated that anti-Tax appeared at about 10 months after transfusion in this patient [25], consistent with results of the appearance of anti-Tax in other carriers [24]. Interestingly, we also previously reported there was an absent antibody response to the neutralizing epitope of HTLV-I that presumably would confer protective immunity against HTLV-I–associated disease [25].

The high HTLV-I antibody titers observed 2 years prior to symptom onset were consistent with the hypothesis proposed by Usuku et al. [26], that HAM/TSP patients have heightened immune responsiveness, which is also characterized by high spontaneous lymphocyte proliferative response linked to specific human leucocyte antigen (HLA) haplotypes. Alternatively,

Jeffrey et al. [27] have proposed that certain alleles, such as HLA-A*02, are associated with protection from HAM/TSP owing to reduction in proviral load in asymptomatic carriers. Thus a specific immunogenetic background, as demonstrated by data from several endemic areas [28], may be predictive of the host's ability to control the severity of infection, which correlates with risk of HAM/TSP or ATL. These data have also been supported by animal experiments, which have shown susceptibility differences to infection among strains of inbred rats, as evidenced by varying levels of antibody response and proviral integration sites in the host genome [29]. The fact that our patient had low proviral load but high antibody titer at the onset of disease probably indicates that the central nervous system is the most important site of events in the pathogenesis of HAM/TSP. Several studies of HAM/TSP patients have shown that antibody titer and viral load are elevated in the central nervous system compared to the peripheral circulation [30]. The presence of elevated antibody titer but low proviral load prior to disease onset may also indicate that titer is a more important predictor of disease progression than proviral load among HAM/TSP patients. The high antibody titer with onset of disease may be explained by several proposed mechanisms of neuronal damage, including autoimmunity [31], direct tissue destruction caused by HTLV-I infection as a result of CD8⁺ HTLV-I-specific cytotoxic T lymphocytes [30], or (less likely) a possible neurotropic HTLV-I viral variant [32].

The different clinical outcomes observed for transfusion recipient RE3013 and RE1659, despite the presence of elevated proviral levels and antibody titers in both, was probably attributable to several factors. First, after transfusion, the development of HAM/TSP has a latency between infection and disease onset that varies from several weeks to years [33]. Thus longer follow-up might have shown that RE1659 may also have developed HAM/TSP. Second, RE3013, owing to her underlying diagnosis, was probably progressively immune compromised. This may have provided a milieu for a more adverse immunologic response with pathogenic properties. Finally, other measures of immune status, such as functional activity of HTLV-I-specific CD8⁺ cytotoxic T cells, may be a better determinant of carriers at risk for development of HAM/TSP [27].

In cross-sectional studies, other investigators have demonstrated the importance of proviral load in HTLV-I infection and related disease. HAM/TSP patients had higher proviral load than did asymptomatic carriers. High levels appeared to be important in identifying patients with recent onset of disease, because the quantity of detectable proviral DNA has been shown to decrease in spinal cord lesions of patients with longer duration of illness [34]. Most of these studies used serial dilutions of sample DNA to estimate semiquantitative proviral DNA levels [35]. In this study and the report by Nagai et al. [36], automated real-time PCR technology was applied to determine proviral DNA load. A direct comparison of the 2 meth-

ods reveals similar results between patient groups with minimum levels of detection of 1–10 copies/10⁵ lymphocytes. In this study and the other cited studies [35, 36], median proviral load ranged from 5–14 copies/100 lymphocytes among HAM/TSP patients and from 0.3–1.6 copies/100 lymphocytes among carriers.

In our comparative analysis of prevalent carriers, ATL and HAM/TSP patients, we also evaluated the utility of proviral load and antibody titer level for distinguishing carriers at risk for HTLV-I-related disease. Proviral load did distinguish HTLV-I carriers from ATL and, in most instances, from HAM/TSP patients. Proviral load also was able to discern ATL from HAM/TSP patients, with the majority of ATL patients having levels well above the 75th percentile of HAM/TSP values. Antibody titer levels were equally successful in distinguishing carriers from ATL and HAM/TSP patients. ATL and HAM/TSP patients had similar titer levels, although the majority of HAM/TSP levels were well above the 75th percentile of ATL values.

In summary, we have delineated the biologic and virologic patterns of HTLV-I in early infection, the carrier state, and disease. Whether or not proviral load can predict development of HAM/TSP or ATL among carriers requires additional study. However, we are optimistic that prospective measurement of antibody titers in combination with HTLV-I proviral load and other biomarkers will have importance in identifying carriers at highest risk for related disease.

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