Identification of Human T-lymphotropic Virus Type I (HTLV-I) Subtypes Using Restrited Fragment Length Polymorphism in a Cohort of Asymptomatic Carriers and Patients with HTLV-I-associated Myelopathy/tropical Spastic Paraparesis from São Paulo, Brazil

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Although human T-lymphotropic virus type I (HTLV-I) exhibits high genetic stability, as compared to other RNA viruses and particularly to human immunodeficiency virus (HIV), genotypic subtypes of this human retrovirus have been characterized in isolates from diverse geographical areas. These are currently believed not to be associated with different pathogenetic outcomes of infection. The present study aimed at characterizing genotypic subtypes of viral isolates from 70 HTLV-I-infected individuals from São Paulo, Brazil, including 42 asymptomatic carriers and 28 patients with HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), using restricted fragment length polymorphism (RFLP) analysis of long terminal repeat (LTR) HTLV-I proviral DNA sequences. Peripheral blood mononuclear cell lysates were amplified by nested polymerase chain reaction (PCR) and amplicons submitted to enzymatic digestion using a panel of endonucleases. Among HTLV-I asymptomatic carriers, viral cosmopolitan subtypes A, B, C and E were identified in 73.8%, 7.1%, 7.1% and 12% of tested samples, respectively, whereas among HAM/TSP patients, cosmopolitan A (89.3%), cosmopolitan C (7.1%) and cosmopolitan E (3.6%) subtypes were detected. HTLV-I subtypes were not statistically significant associated with patients' clinical status. We also conclude that RFLP analysis is a suitable tool for descriptive studies on the molecular epidemiology of HTLV-I infections in our environment.

Key words: HTLV-I - subtype - restricted fragment length polymorphism - São Paulo - Brazil

Human T-cell lymphotropic virus type I (HTLV-I) is a human retrovirus that belongs to the genus Deltaretrovirus (Poiesz et al. 1980, Murphy 1996). Even though most infected individuals remain asymptomatic throughout their lives, about 1 to 5% may progress to disease development (Mahieux et al. 1997). HTLV-I-associated illnesses include adult T-cell leukemia/lymphoma (ATLL) (Takatsuki et al. 1985) and HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Gessain et al. 1985, Osame et al. 1986), as well as inflammatory syndromes such as uveitis (Mochizuki et al. 1994), arthropathy (Nishioka et al. 1989), polymyositis (Morgan et al. 1989), Sjögren's syndrome (Mariette et al. 1995) and infective dermatitis (La Grenade et al. 1990).

HTLV-I infection is distributed worldwide with particularly high seroprevalence being detected in Japan (Morofuji-Hirata et al. 1993), Southeastern USA (Canavaggio et al. 1990), the Caribbean region (Wattel et al. 1992), Central and South America (Trujillo et al. 1992, Segurado et al. 1997a), as well as Central Africa (Mahieux et al. 1997) and the Melanesian islands (Yanagihara et al. 1990).

In Brazil HTLV infections can be considered endemic (Cortes et al. 1989, Bellei et al. 1996, Ferreira Junior et al. 1995, Casseb et al. 1997, Dourado et al. 1998) and many individuals have been recognized as HTLV-I/II-infected since compulsory screening of blood donors was started in 1993. Among Brazilian asymptomatic blood donors HTLV seroprevalence has been shown to range from 0.3% in São Paulo (Segurado et al. 1997b) to 1.3% in Salvador, Bahia (Galvão-Castro et al. 1997). Likewise HTLV-I-associated disease, including ATLL and HAM/TSP, has been described in different areas of the country (Castro et al. 1989, Oliveira et al. 1990, Araújo et al. 1999).

Genetic stability among human immunodeficiency virus (HTLV) isolates is high. In contrast to HIV *env* sequences, that may present up to 30% genetic diversity, HTLV-I genotypic variability is usually under 4% (Ratner et al. 1991). Detailed analysis of the proviral genome demonstrates larger variability in the long terminal repeat (LTR) and *env* genes, whereas *gag* and *pol* sequences seem to present higher similarity among different samples. For this reason investigators have most often used amplification of LTR sequences in order to characterize particular genotypic subtypes (Komurian-Pradel et al. 1992). These isolates exhibit peculiar geographic distributions

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(Schulz et al. 1991), but are believed not to be associated with different pathogenetic outcomes of these retroviral infections (Ureta-Vidal et al. 1994).

The present study thus aimed at characterizing HTLV-I subtypes among asymptomatic carriers of these retroviral infections and in patients with HAM/TSP from São Paulo, and to investigate whether different molecular subtypes were associated with disease development in these individuals.

MATERIALS AND METHODS

Patient selection and data collection - Study subjects were selected from a cohort of 42 HTLV-I asymptomatic carriers, identified at voluntary blood donations, that was followed at the Infectious Diseases outpatient clinic of the Hospital das Clínicas, University of São Paulo Medical School, from 1993 to 1998 and from 28 HAM/TSP patients, followed at the Neurology outpatient clinic of the same institution during the same period of time. The cohort of HTLV-I asymptomatic carriers included 25 men (59.5%) and 17 women (40.5%). Their ages ranged from 18 to 58 (mean 39.8 and median 40). Neurologic patients presented chronic progressive myelopathy and met World Health Organization (WHO) criteria for the diagnosis of HAM/TSP (WHO 1989). Among HAM/TSP patients there were 16 women (57.1%) and 12 men (42.9%), with ages ranging from 27 and 74 (mean 54.5 and median 51.5). After having signed informed consent, patients were interviewed by members of the investigators' team in search of demographic and epidemiological data, information on potential exposure to retroviral infection and previous personal and familial morbidity. Patients were also submitted to a detailed clinical examination and to blood collection for laboratory investigation. The study protocol was approved by the Institutional Review Board of the University of São Paulo Medical School.

Serodiagnosis of HTLV infection - The diagnostic algorithm in the present study included seroscreening for anti-HTLV-I/II antibodies, using enzyme immunoassays (Hemobio HTLV-I/II®, Embrabio, São Paulo, Brazil or HTLV-I+IIGE80/81®, Murex Diagnósticos, São Paulo, Brazil). Seropositive samples were then confirmed by Western blot testing (HTLV Bolt 2.4®, Diagnostic Biotechnology, Singapore), allowing differentiation between HTLV-I and HTLV-II infections, based on seroreactivity to type-specific recombinant peptides, according to manufacturers' instructions. Following international recommendations (WHO 1990), samples were considered HTLV-I-seropositive whenever reactivity was demonstrated to both *gag* (p24 and/or p19) and *env* (gD21, gp 46 and rgp46-I) viral antigens.

Molecular confirmation of HTLV-I infection - HTLV-I proviral genome sequences were sought after by nested PCR amplification from peripheral blood mononuclear cells (PBMC), using consensus primers for the *tax* region (1st round – SK43 and SK44; 2nd round – nt 7264-7283 and 7501-7485). Differentiation between HTLV-I and HTLV-II infections was based on restricted fragment length polymorphism (RFLP) analysis of nested PCR products, using *Taq*-1, as previously described (Tuke et al. 1992).

HTLV-I genotypic subtyping - LTR proviral sequences were amplified by nested PCR from patients' PBMC, using a standardized protocol, modified from the original description by Komurian-Pradel et al. (1992). A hot-start PCR was performed that used for the lower solution (27.7 µl): 0.5 µl of each 25 µM primer, 2 µl of 2.5 µM dNTP mix, 2.5 µl of PCR buffer (0.1 M Tris-HCl, pH 8.3, 0.5 M KCl, 1 mg/ml gelatin and 2.5 µl of 50 mM Mg Cl_2) and for the upper solution (26 µl): 2 µl of DNA template from tested samples (~0.8-1.6 µg); 2.5 µl of PCR buffer and 1.25 units of *Taq pol*). After initial incubation at 95ºC for 5 min, samples underwent 25 amplification cycles, using 95ºC for 20 sec, 72ºC for 20 sec and a final extension step of 7 min. First round primers were SPL2R – nt 727-748 (5'CGC TCG CTG TCG GGT AGG ATA T3') and SPL3F – nt 23-52 (5'AC AAT GAC CAT GAG CCC CAA ATA TCC CCG G3'). 10 µl of PCR products were then submitted to a 2nd amplification, with 1 µl of each 25 µM primer, 4 µl of 2.5 mM d NTP mix, 10 µl of PCR buffer, 5 µl of 50 mM $MgCl_2$ and 2.5 units of *Taq pol*, in a total volume of 100 µl per sample, following the same protocol used in the first round. Second round primers were: SPL2R and SPL2F – nt 31-53 (5'ACC ATG AGC CCC AAA TAT CCC CC3'). Molecular subtyping of amplified LTR sequences was obtained after digestion of the 718 bp nested PCR product by a panel of restriction endonucleases that included: *Apa*I (Sigma), *Nde*I (Promega), *Dra*I (Sigma), *Sac*I (Promega) or *Sst*I (Sigma) and *Mae*III (Boehringer-Mannheim). In this procedure we used 10 µl of amplicons, 2 µl of enzyme buffers, 10 units of each enzyme and water in a total volume of 20 µl for each sample. Incubation lasted for 3 h and was carried out at 55ºC for *Mae*III, 30ºC for *Apa*I and 37ºC for the other enzymes. RFLP analysis was performed after electrophoresis in ethidium bromide-stained 2% agarose gels.

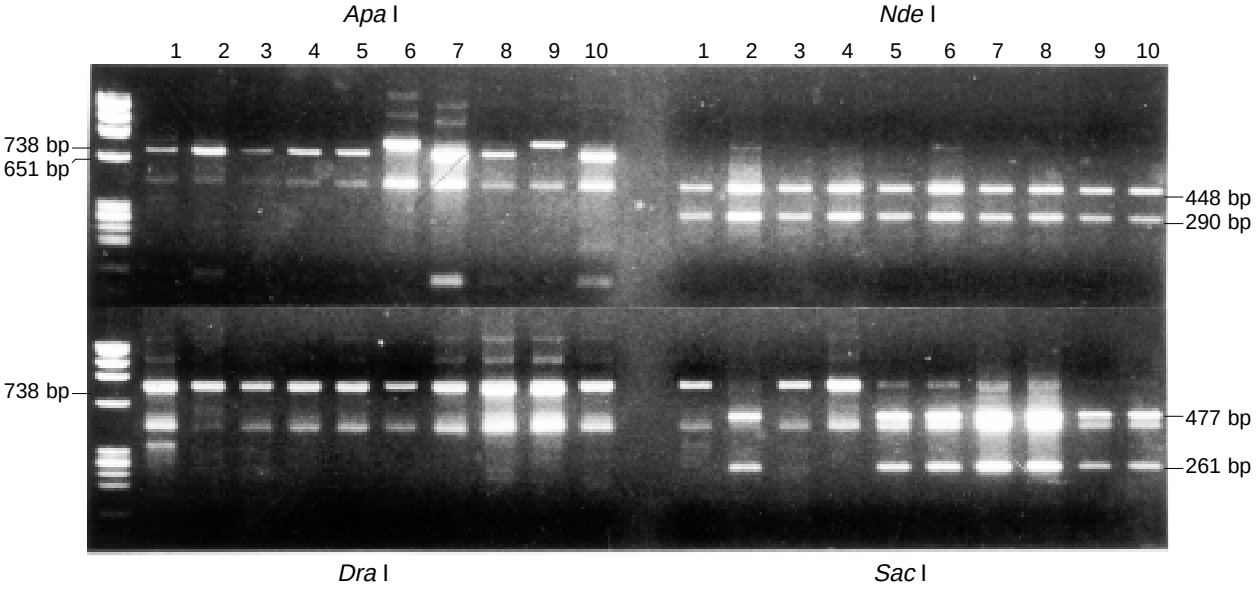
Data analysis - Frequencies of different HTLV-I genotypic subtypes were verified and their association with clinical status (asymptomatic versus HAM/TSP) was analyzed, using Fisher's exact test with $\alpha = 0.05$.

RESULTS

HTLV-I genotypic subtyping - RFLP analysis of amplified LTR sequences of the proviral genome (Figure) identified four distinct viral subtypes in the cohort, as detailed in the Table. Cosmopolitan A was the most prevalent HTLV-I subtype, accounting for 31/42 (73.8%) of infections in asymptomatic carriers and for 25/38 (89.3%) among HAM/TSP patients. Blood donors infected with cosmopolitan B, cosmopolitan C and cosmopolitan E subtypes, as well as myelopathic patients infected with cosmopolitan C and cosmopolitan E viral subtypes were also identified. HTLV-I genotypic subtypes were shown not to be associated to patients' clinical status (Fisher's exact test, $p = 0.33$).

DISCUSSION

The present study has characterized HTLV-I molecular subtypes that are responsible for infection of a cohort of 42 HTLV-I-seropositive asymptomatic carriers, as well as 28 HAM/TSP patients, followed at the Infectious Diseases and Neurology outpatient clinics of the University of São Paulo Medical School Hospital from 1993 to 1998.



Ethidium bromide-stained 2% agarose gel containing human T-lymphotropic virus type I (HTLV-I) long terminal repeat (LTR) proviral sequences, submitted to digestion by *Apa*I, *Nde*I, *Dra*I and *Sac*I. Left lane: bp ladder, lanes 1-10: LTR proviral sequences from 10 selected HTLV-I asymptomatic carriers. Sequence sizes are shown on both margins.

TABLE

Human T-lymphotropic virus type I (HTLV-I) genotypic subtyping, based on restricted fragment length polymorphism (RFLP) analysis of long terminal repeat (LTR) sequences of proviral DNA, amplified by nested PCR from peripheral blood mononuclear cells of 42 asymptomatic carriers and 28 HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) patients, São Paulo, Brazil (1993-1998)

RFLP profile of HTLV-I LTR sequences					HTLV-I subtype [a]	Asymptomatic carriers		HAM/TSP patients	
*Apa*I	*Nde*I	*Mae*III	*Dra*I	*Sac*I or *Sst*I		n	%	n	%
+ [b]	+	- [c]	-	+	Cosmopolitan A	31/42	73.8	25/28	89.3
+	+	-	+	-	Cosmopolitan B	3/42	7.1	0/28	0
+	+	-	-	-	Cosmopolitan C	3/42	7.1	2/28	7.1
-	+	-	-	+	Cosmopolitan E	5/42	12	1/28	3.6

a: classification of HTLV-I genotypic subtypes as proposed by Ureta-Vidal et al. (1994); *b*: presence of restriction site for corresponding endonuclease in LTR sequences; *c*: absence of restriction site for corresponding endonuclease in LTR sequences

Among all HTLV-I-infected subjects, cosmopolitan A was the most prevalent viral subtype, regardless of their clinical status. It was shown to account for 73.8% of infections among asymptomatic carriers and for 89.3% among myelopathic patients. This observation is concordant with previous evidence that indicates this viral subtype to be the most widespread of HTLV-I genotypic variants (Ureta-Vidal et al. 1994, Yamashita et al. 1996). In a previous study of 27 Brazilian HTLV-I asymptomatic carriers and patients with HAM/TSP from different parts of the country, cosmopolitan A subtype was also shown to predominate (Yamashita et al. 1999).

However infections due to other HTLV-I variants were also demonstrated in our cohort: cosmopolitan C and cosmopolitan E subtypes were seen in both clinical situations (7.1% and 12%, respectively, among asymptomatic carriers and 7.1% and 3.6% in HAM/TSP patients), whereas infections by HTLV-I cosmopolitan B subtype were identified in 7.1% of asymptomatic carriers, exclusively.

It should be pointed out that cosmopolitan E subtype exhibits a RFLP profile in LTR proviral sequences that closely resembles HTLV-I cosmopolitan A isolates. They both differ just by a loss of *Apa*I restriction site at nucleotide 113 in HTLV-I cosmopolitan E samples. Therefore it is reasonable to suppose that a single point mutation, located at the corresponding restriction site, may have originated this viral subtype from HTLV-I cosmopolitan A strains, the most widespread of all genotypic variants. Further studies based on nucleotide sequencing of proviral DNA may help clarify this evolutionary issue.

In this context HTLV-I cosmopolitan C isolates, also demonstrated among asymptomatic carriers and HAM/TSP patients in this study, were originally described in infected populations from West Africa and the Caribbean basin (Miura et al. 1994). This variant seems to be ethnically associated with black race and its peculiar geographical distribution is believed to be consequent to human migrations related to African slavery trade into colonies

on the American continent. In our cohort this HTLV-I genotypic subtype was indeed detected in a black female asymptomatic blood donor, but also in a white HBV- and HIV-coinfected HAM/TSP patient, who reported previous intravenous drug use (IDU) with shared needles and syringes. Therefore contamination from a human source of African descent could not be ruled out in this case. However cosmopolitan C isolates were then also identified in two white asymptomatic HTLV-I carriers with no apparent risk factor for retroviral exposure. A more in-depth epidemiological investigation would have been necessary to clarify the origin of infection by such a viral subtype in these individuals.

HTLV-I cosmopolitan B subtype was found in three asymptomatic carriers in our cohort. Two of them descended from Japanese immigrants, who came from Hokkaido and Kyushu islands, located in the extreme northern and southern parts of that Asian country, respectively. In fact HTLV-I cosmopolitan B strains are well known to present a very particular geographical distribution, that is limited to those specific Japanese regions and this has been attributed to the ancestral ethnic origins of those populations (Miura et al. 1994). Similar findings were reported by Yamashita et al. (1999) among Brazilian HTLV-I-infected individuals of Japanese descent. The remaining HTLV-I cosmopolitan B-infected subject in our cohort was an asymptomatic blood donor, who reported having received previous blood transfusions, during gastric surgery, as the only recognizable risk factor for retroviral exposure. Unfortunately a retrospective investigation in search of his blood donors was not feasible, so as to clarify the origin of this HTLV-I infection.

The fact that identified HTLV-I subtypes were not associated with patients' clinical status is concordant with results from previous investigations (Komurian-Pradel et al. 1992, Ureta-Vidal et al. 1994). So far there is no conclusive evidence that particular HTLV-I genotypic variants are more pathogenic or lead to increased risk of disease development.

Some comments however should be made on potential limitations of the present study. The fact that patient inclusion sites were restricted to reference centers for HTLV counseling and care may have caused selection bias. The cohort may thus not be representative of the HTLV-I-infected population from São Paulo. However the lack of population-based serosurveys of HTLV infection in our country precludes a more comprehensive approach in this regard. We should admit the external validity of this study to be limited to similar series of HTLV-I-infected individuals.

It is also critical to mention certain intrinsic drawbacks of the molecular biology methods used in the present investigation. Even though nucleotide sequencing of the proviral genomes, followed by comprehensive phylogenetic analysis is considered the gold standard method in molecular epidemiological studies of viral infections, RFLP analysis usually yields reliable results that are highly concordant with those obtained by DNA sequencing, as pointed out by Ureta-Vidal et al. (1994).

This study therefore presents a RFLP analysis of HTLV-I proviral genomes as a suitable investigation approach for descriptive studies on the molecular epidemiology of these retroviral infections and provides a contribution to a better understanding of their behavior in our environment.

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