Molecular characterization of two isolates of human T cell leukaemia virus type II from Italian drug abusers and comparison of genome structure with other isolates

Davide Zella, Ada Cavicchini, Marco Salemi, Claudio Casoli, Franco Lori, Giorgio Achilli, Ercole Cattaneo, Viviana Landini and Umberto Bertazzoni

Istituto di Genetica Biochimica ed Evolutiva del C.N.R., Via Abbiategrasso 207, 1-27100 Pavia, Agrimont, Massa Carrara, University of Parma, Italy, Laboratory of Tumor Cell Biology, National Cancer Institute, Bethesda, Maryland, U.S.A. and Clinica Malattie Infettive, IRCCS Policlinico S. Matteo, Pavia, Italy

The human T cell leukaemia virus type II (HTLV-II), whose pathogenicity is as yet unclear, was recently found to be associated with intravenous drug abuse in North America and Europe. HTLV-II was isolated from two Italian drug abusers belonging to the same cohort and coinfected with human immunodeficiency virus type 1. Two new isolates, HTLV-II Gu and Va, were established in a culture of BJAB cells, a continuous B cell line (Epstein-Barr virus-negative), and characterized by nucleotide sequence analysis of the long terminal repeat (LTR) and portions of the gag, env and X regions. These sequences were compared to those of the HTLV-II Mo isolate reported in the literature. No major variations were observed in important regulatory elements of LTR nor in the stem–bulge–loop configuration known to be essential for binding of rex protein. The results obtained from the sequence of the 1988 nucleotides examined indicated a 1–6% variability between the Gu and Va isolates and about 6% with respect to Mo. Notable differences were found in the structure of putative open reading frames of the X region when compared to those reported for the Mo isolate. Restriction analysis of proviral DNA of two isolates and comparison with the physical map of the Mo isolate confirmed the existence of genetic heterogeneity in the HTLV-II group and demonstrated that the new isolates Gu and Va belong to the HTLV-IIb subtype. The results of this study show that the new isolates have distinct features with respect to the Mo isolate though all important regulatory elements of the LTR appear to be well conserved.

Introduction

The human T cell leukaemia (or lymphotropic) virus type I (HTLV-I) and type II (HTLV-II) are closely related oncogenic retroviruses (Cann & Chen, 1990; Hjelle, 1991). HTLV-I has been firmly associated with adult T cell leukaemia (Poiesz et al., 1980; Yoshida et al., 1982) and with a chronic degenerative neurological disease, tropical spastic paraparesis/HTLV-I-associated myelopathy (Gessain et al., 1985; Osame et al., 1986).

HTLV-II has been linked with unusual rare cases of hairy cell leukaemia (Kalyanaraman et al., 1982; Rosenblatt et al., 1986). Recently, HTLV-II has been demonstrated in a significant proportion of intravenous drug users (IVDUs) in the United States and in Europe (Tedder et al., 1984; Robert-Guroff et al., 1986; Lee et al., 1989; Ehrlich et al., 1989; Kwok et al., 1990; Page et al., 1990; Zella et al., 1990; Khabbaz et al., 1991; Varnier et al., 1991), as well as among U.S. blood donors (Hjelle et al., 1990; Lee et al., 1991; Hjelle & Chaney, 1992). Endemic HTLV-II infection has been reported among Guaymi Indians in Panama (Lairmore et al., 1990).

Only a few isolates of HTLV-II have been reported (Kalyanaraman et al., 1982, 1985; Rosenblatt et al., 1986) and, to our knowledge, none has been characterized in Europe to date. In a previous study, we screened a cohort of Italian IVDUs by serological and PCR analyses and found a much higher frequency of infection with HTLV-II than with HTLV-I (Zella et al., 1990). In order to confirm these observations, we have attempted to isolate the virus from peripheral blood mononuclear cells (PBMCs) of IVDUs co-infected with human immunodeficiency virus (HIV-1) and HTLV-II. Co-cultivation of PBMCs with BJAB, a B cell line not susceptible to HIV-1, was recently used to isolate HTLV-II from IVDUs concomitantly infected with HIV-1 (Hall et al., 1990).

This study describes the establishment in culture of two new HTLV-II isolates by co-cultivation with BJAB cells and their characterization by sequence analysis of
the long terminal repeat (LTR) and portions of the gag, env and X regions of proviral DNA. We have studied the variability between the two isolates and with respect to the reported sequence of the HTLV-II–Mo isolate (Shimotohno et al., 1984, 1985). The mutations occurring in the three different regions of the LTR (U3, R and U5) of the new isolates were examined with regard to previously reported important elements and the possible secondary structures of viral RNA recognized by the regulatory proteins.

In a recent study, the physical maps of new isolates of HTLV-II from Northern American IVDUs were compared with that of the Mo isolate and the existence of two closely related molecular subtypes of HTLV-II designated HTLV-IIa and HTLV-IIb was observed (Hall et al., 1992). In order to extend these observations we have further characterized our HTLV-II isolates by restriction analysis and compared the map patterns obtained with that of other HTLV-II isolates.

Methods

Virus isolation. Fresh PBMCs of IVDUs, belonging to the cohort previously studied and coinfected with HIV-1 and HTLV-II (Zella et al., 1990) were separated on Ficoll/Hypaque. Mononuclear cells were cultivated for 48 h in growth medium (RPMI-1640, 20% calf serum, 1% t-glutamine), supplemented with 5 μg/ml phytohaemagglutinin (Sigma), followed by culture in growth medium supplemented with 10% interleukin-2 (IL-2) (Cellular Products). The initial PBMC cultures were co-cultivated in growth medium without supplements with an established B cell line (BJAB; Klein et al., 1974) which is not susceptible to HIV-1 and was shown by Hall et al. (1990, 1992) to support the replication of HTLV-II. Infection was monitored by syncytium formation and by assaying Mg2+-dependent reverse transcriptase (RT) in culture supernatants. Indirect immunofluorescence with an established B cell line (BJAB; Klein et al., 1974) was performed as described above for the first symmetric step, except that 40 cycles were used. DNA sequencing was carried out using the ddNTP chain termination method (T7 sequencing kit, Pharmacia). Primers used were: M13-20 forward; T7; nt 6612 to 6641; nt 6677 to 6706 of the isolate Mo (Shimotohno et al., 1985).

Southern hybridization analysis. DNA was isolated from the BJAB cells using phenol/chloroform extraction and then digested with restriction endonucleases (Boehringer Mannheim) according to the manufacturers’ instructions and as described later in Fig. 4. Samples were electrophoresed on 0.8% agarose gels, transferred to nylon membranes (GeneScreen, NEN Research Products) and hybridized with 32P-labelled random-primed probes (Amersham). Two HTLV-II probes, 3' (3.5 kb) and 5' (4.7 kb), each defined by BamHI sites and which together covered the entire HTLV-II-Mo provirus, were employed (Gelmann et al., 1984). After hybridization, membranes were washed twice with 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) containing 0.1% SDS for 15 min, and then washed with 0.1× SSC containing 0.1% SDS at room temperature for 20 min and at 60 °C for 45 min. Membranes were exposed to Fuji AR film with intensifying screens at ~70 °C for 16 h.

Results

Virus isolation

In order to obtain HTLV-II isolates from individuals co-infected with HIV-1, it is necessary to overcome the cytopathic effect of HIV-1, which is responsible for continuous cell death. This can be achieved by co-cultivating the PBMCs with a cell line that does not have CD4 receptors and thus cannot support the replication of HIV-1 but can be infected by HTLV-II. As suggested by Hall et al. (1990, 1992), the initial PBMC cultures obtained from two dually infected drug abusers were co-cultivated with BJAB cells, a continuous Epstein–Barr virus-negative B cell line (Klein et al., 1974). Co-cultivation resulted initially in prominent syncytium formation, but upon addition of fresh BJAB cells and continued culture, two permanently growing cell lines
were established. Infection was determined by positive Mg\textsuperscript{2+}-dependent RT activity in culture supernatants (data not shown). It thus appears that HTLV-II replication in BJAB cells is cytopathic, as previously observed by Hall et al. (1992). When the infected BJAB cells were monitored by indirect immunofluorescence using a monoclonal antibody to p24 of HTLV-II, more than 90% of the cells were found to be positive. The two HTLV-II isolates, maintained on BJAB cultures, were designated HTLV-II-Gu and HTLV-II-Va.

### Nucleotide sequence analysis

An analysis of the nucleotide sequence of the LTR region and the 5' portion of gag of proviral DNA (nt 31 to 1080) of the two HTLV-II isolates Va and Gu was performed using a direct PCR amplification procedure, as described in Methods. As shown in Fig. 1, the sequences of the new isolates were compared with that of HTLV-II-Mo published by Shimotohno et al. (1984, 1985) which was used as reference for alignment. It should be noted that the nucleotide sequence numbering does not correspond to that of Shimotohno et al. (1985) starting from nt 160 since the insertions of new bases in the isolates had to be considered.

The percentage variations (including mutations, insertions and deletions) of the two isolates Gu and Va with respect to the published sequence of HTLV-II-Mo (Shimotohno et al., 1984, 1985) are reported in Table 1. A divergence of 3.5% was observed between the two isolates in the U3 region (nt 31 to 318) and of 7 and 8.3% with respect to HTLV-II-Mo. Of the three direct repeats of 21 nt, reported for Mo and comprising nt 90 to 250, only the second (nt 132 to 153) was significantly changed in the two isolates (see Fig. 1). Concerning the three small direct repeats of 7 nt each (located between nt 120 and 170) and the two inverted repeats (nt 280 to 310, matching with inverted repeats in the R region), only a few variations were noted in the two isolates with respect to the Mo sequence. As shown in Fig. 1, the variability between the two isolates is low since the same type of change with respect to the Mo isolate was usually found. Both the poly(A) signal (nt 270 to 280) and TATA box (nt 290 to 300) showed no functional changes in the two isolates. Also the sequence of the RNA cap site (nt 316 to 322) was conserved in the two isolates.

Concerning the R region (nt 320 to 568), six changes with respect to the Mo isolate were observed in the Gu and Va isolates, corresponding to a variability of 6.5% (Table 1). Within the splice donor site, a single mutation (A to G at nt 453) was observed in the Gu and Va isolates (see Fig. 1). The sequence following the splice donor site (nt 460 to 500), which is particularly important in the recognition of rex protein (Black et al., 1991; Kim et al., 1991), contained only one mutation (C to T at nt 487) in the two isolates. The difference between the Gu and Va isolates in the whole R region was restricted to 2 nt, corresponding to a variability of 0.8% (see Table 1).

A variability of 4.8% was observed in the U5 region (nt 569 to 777) between the two new isolates and of 8.7% using a monoclonal antibody to p24 of HTLV-II, more than 90% of the cells were found to be positive. The two HTLV-II isolates, maintained on BJAB cultures, were designated HTLV-II-Gu and HTLV-II-Va.

### Table 1. Percentage variability between nucleotide sequences of HTLV-II-Mo and new isolates HTLV-II-Gu and HTLV-II-Va

<table>
<thead>
<tr>
<th>Region</th>
<th>Nt</th>
<th>Mo/Gu</th>
<th>Mo/Va</th>
<th>Gu/Va</th>
</tr>
</thead>
<tbody>
<tr>
<td>U3</td>
<td>31-319</td>
<td>7.0</td>
<td>8.3</td>
<td>3.5</td>
</tr>
<tr>
<td>R</td>
<td>320-368</td>
<td>6.5</td>
<td>6.5</td>
<td>0.8</td>
</tr>
<tr>
<td>U5</td>
<td>569-777</td>
<td>8.7</td>
<td>8.7</td>
<td>4.8</td>
</tr>
<tr>
<td>LTR</td>
<td>31-777</td>
<td>7.3</td>
<td>7.8</td>
<td>3.0</td>
</tr>
<tr>
<td>gag</td>
<td>821-1080</td>
<td>5.4</td>
<td>5.0</td>
<td>0.4</td>
</tr>
<tr>
<td>env</td>
<td>6209-6637</td>
<td>4.0</td>
<td>3.8</td>
<td>0.8</td>
</tr>
<tr>
<td>X</td>
<td>6638-7192</td>
<td>6.5</td>
<td>6.5</td>
<td>0.3</td>
</tr>
<tr>
<td>Total</td>
<td>1988</td>
<td>6.1</td>
<td>6.2</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Fig. 1. Variations in the nucleotide sequences of LTR and gag regions of HTLV-II–Gu and –Va isolates with respect to the Mo isolate. The HTLV-II–Mo sequence published by Shimotohno et al. (1985) was used for alignment. Deletions are indicated by a — sign in the Gu and Va isolates and insertions by a — sign in the Mo sequence and by the indicated bases in the new isolates. A blank site indicates no variation with respect to Mo. The amino acid changes in the new isolates are indicated by the capital letter in parentheses. Sequences analysed extend from nt 31 to 1080. The numbering system appears to differ from that of Shimotohno et al. (1985) after nt 160 since the insertions of new bases in the isolates had to be considered.
with respect to HTLV-II-Mo, as reported in Table 1. The poly(A) site (nt 565 to 571) was found to be strictly conserved in the Gu and Va isolates. It appears that the number of variations occurring in U5 between the new isolates was much higher than that observed in the R region, and the majority of these were located within nt 640 to 680, a region that can be deleted without affecting the binding activity of rex protein (Black et al., 1991) (see Fig. 1). The primer binding site (nt 780 to 797) was very well conserved since no changes were found in the two isolates with respect to Mo.

The RNA secondary structure maps of HTLV-II-Mo and of the isolate Gu, using sequences from the cap site to the end of U5 (nt 320 to 780), were generated by computer analysis, using software which maximizes the stability of the structure (Fig. 2). Although the structures of Mo and Gu appear to be different, the region recognized by rex, that is the rex-responsive element

Fig. 2. RNA secondary structures of HTLV-II-Mo (a) and HTLV-II-Gu (b) from the cap site to the end of U5, generated by a Vax computer, using the University of Wisconsin DNA analysis software. The sequence of Shimotohno et al. (1984) was used as a reference.
Characteristics of new isolates of HTLV-II

Env and X regions of HTLV-II–Gu and –Va isolates with respect to the Mo isolate (Shimotohno et al., 1985) which was used for alignment. Sequence of IIb isolates was from Hall et al. (1992). Sequences extend from nt 6209 for Gu and nt 6267 for Va up to nt 7192 for both isolates. The nucleotide sequence numbering appears to differ from that of Shimotohno et al. (1985) from nt 6824 (see also the legend of Fig. 1). ns, Not sequenced.

The nucleotide sequence of the 3' portion of env (nt 6209 to 6637) with respect to the Mo isolate and with respect to the sequence of subtype IIb (Hall et al., 1992) are reported in Fig. 3. The number of changes observed accounted for a variability of 0.8% between the two isolates and of about 4% with Mo (see Table 1). As already observed for gag, the great majority of the modifications observed for the two isolates with respect to Mo were point mutations of the same type and were located in the third position of the codon, producing only two amino acid changes in Gu and three in Va (Fig. 3).

Concerning the 5' portion of the X region (nt 6638 to 7192), only two base changes were noted between the two isolates (0.3% divergence) whereas a variability of 6.5% with Mo was observed (see Fig. 3 and Table 1). The insertion of a C at position 6824 (recently reported also for the Mo isolate by Ciminale et al., 1992) and two deletions, one at position 6752, found also for Mo (Ciminale et al., 1992) and one at position 6940, not reported for the Mo isolate, appear to be important.

The presence of several putative open reading frames (ORFs) in the X region (Shimotohno et al., 1985; Ciminale et al., 1992) was confirmed also for the new isolates. Two ORFs appear to be of particular interest: RF1A extending from nt 6807 to nt 7172 and RF2A, from nt 6511 to 6840. The first ORF starts in association with a splice donor site, as recently shown by Ciminale et al. (1992) but appears to be considerably longer. Of the two other splice sites observed by these authors, that at nt 6844 was maintained whereas that at nt 6827 was not.

Provirus restriction analysis

In order to obtain a physical map of the new isolates for comparison with those of the Mo and NRA isolates (Gelmann et al., 1984; Rosenblatt et al., 1986) and with those of other HTLV-II isolates recently reported by Hall et al. (1992), chromosomal DNA from infected BJAB cultures was digested with several restriction enzymes and hybridized with the two 5' and 3' probes (see Methods). The results are summarized in Fig. 4. Restriction analysis of Gu and Va with BamHI resulted in the detection of an additional site in the 5' end, not found for Mo isolate. Furthermore, when Va and Gu proviral DNA was restricted with SacI, PstI and BglII, the corresponding sites in the 5' end were not observed (Fig. 4). These data are consistent with the existence of two molecular subtypes of HTLV-II: one similar to
Published sequence of Shimotohno isolates was determined by Southern blot hybridization analysis after digestion of proviral DNA with the endonucleases indicated (see Methods). Differences between isolates Gu and Va with respect to Mo and Gu isolates (b) with that of the Mo isolate (a) constructed from the sequence analysis. The CRS, located between R and U5 (Black et al., 1991), showed rather more variations in the Gu and Va isolates with respect to Mo although these were of the same type, as noted in the R region. However, the CRS secondary structure map for the Gu and Va had a stem–bulge–loop structure (at nt 570 to 620) which is very similar to that found for the Mo isolate. The variations observed in the 5' portion of the U5 region were particularly numerous. Thus it appears that this region could tolerate a higher number of mutations and that the variations, which could change RNA folding, did not affect the viability of the virus. In contrast, the sequence of the primer binding site, just after the 3' end of U5, appeared to be exactly conserved in all the different sequences examined.

Concerning the 5' portion of gag, the variations occurring in this region indicate that the Gu and Va isolates differed from Mo in the same positions and that the same type of mutation was occurring, including a single amino acid change. A similar distribution was also observed for the sequence of the 3' portion of env since the variability between the two isolates was negligible and the majority of mutations were located in the third position of the codon, producing very few amino acid changes with respect to the Mo isolate. In a recent paper,
Hall et al. (1992) presented evidence for the existence of two closely related molecular subtypes of HTLV-II, one with a physical map identical to that of the Mo isolate and designated IIa, and the second being closer to the NRA isolate and named IIb. The comparison of the sequence of the 3' end of env of our isolates with that of the HTLV-IIb subtype showed that exactly the same type of mutations were found in IIb and Gu with respect to Mo and only very few changes were observed between IIb and Va. This would suggest that our isolates belong to the HTLV-IIb subtype.

Sequencing of the X region from the 3' env terminator to the 5' tax demonstrated that the two isolates were almost identical (only 0.3% divergence) whereas they differed from the Mo isolate (6.5% variability). The same putative ORFs were found in our isolates and were notably different, both in length and in predicted amino acid composition, from those reported for Mo by Ciminale et al. (1992). In particular, of the three splice sites observed by these authors, one was lacking in our sequence.

The results obtained from the nucleotide sequence indicate that the new Gu and Va isolates, which derive from the same cohort of IVDUs (Zella et al., 1990), are very similar, since they differed in only 33 of the 1988 nt examined (1.6% variability) whereas a variability of about 6% was observed with respect to HTLV-II-Mo isolate.

In order to confirm the data obtained for the env sequence and clarify whether the observed differences of our isolates from Mo could suggest that their molecular structure was that of subtype IIb, restriction analysis of the proviral DNA of the two isolates was carried out. In fact, the physical maps of the isolates Va and Gu were identical (for the restriction enzymes used) to that described for the NRA isolate (Rosenblatt et al., 1986) and those of a group of other isolates that were classified as HTLV-IIb (Hall et al., 1992).

These results appear to be of particular interest in tracing the world-wide distribution and the natural history of this human retrovirus and in the identification of sequences that could be important in designing peptides for developing specific serological assays.

Concerning the origin of these new isolates, since HTLV-II has been recently described as a New World virus (Biggar et al., 1992; Hjelle & Chaney, 1992) it is conceivable that they originate from strains similar to those infecting North American subjects. However, since differences have been observed between our isolates and the prototype Mo, it would be interesting to compare other European and American isolates in order to establish similarity and/or differences.

In conclusion the results of this study show that the two isolates of HTLV-II obtained from Italian IVDUs are very similar to each other and that the structures of all important regulatory elements of the LTR are well conserved. However, the new isolates appear to be distinct from isolate HTLV-II-Mo since they belong to the IIb subtype and have different ORFs in the X region.

We thank Antoine Gessain and Mary Klotman for helpful comments and for critical reading of the manuscript, and Carl Saxinger for computer analysis of the sequences. D. Zella is the recipient of a Hoffman-La Roche post-doctoral fellowship. This work was supported in part by grants from the Istituto Superiore di Sanità, Progetto AIDS 1992.

References


(Received 30 July 1992; Accepted 3 November 1992)