Influence of human immunodeficiency virus type 1 subtype on mother-to-child transmission

Natàlia Tàpia, Sandra Franco, Francesc Puig-Basagoiti, Clara Menéndez, Pedro Luis Alonso, Hassan Mshinda, Bonaventura Clotet, Juan Carlos Saiz and Miguel Angel Martínez

1Fundació irsiCaixa, Hospital Universitari Germans Trias i Pujol, 08916 Badalona, Spain
2,3Liver Unit and Unitat d’Epidemiologia i Bioestadística, Department of Medicine, Institut d’Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Hospital Clinic, Facultad de Medicina, Universidad de Barcelona, Spain
4Ifakara Health Research and Development Centre, Ifakara, Tanzania

The present study was designed to assess whether the subtype of human immunodeficiency virus type 1 (HIV-1) could affect the rate of HIV-1 mother-to-child transmission in a cohort of 31 HIV-1-seropositive pregnant Tanzanian women. In order to assign a subtype to the samples analysed, nucleotide sequencing of the HIV-1 long terminal repeat U3 and C2V3C3 envelope regions was performed from the sera of these 31 pregnant women. Except in three cases, amplification of both regions was achieved in all samples. Subtypes A (n = 13, 46 %), C (n = 6, 21 %) and D (n = 2, 7 %), as well as a number (25 %) of A/C, C/A, D/A and C/D recombinant forms (n = 3, 2, 1 and 1, respectively), were identified. Of the 31 HIV-1 seropositive pregnant women analysed, eight (26 %) transmitted HIV-1 to their infants. Among the eight transmitter mothers, four (4 of 13, 31 %) were infected with HIV-1 subtype A, one (1 of 6, 17 %) with HIV-1 subtype C, none (0 of 2, 0 %) with HIV-1 subtype D and three (3 of 7, 43 %) with HIV-1 subtype recombinant A/C. These findings show no significant differences in the mother-to-child transmissibility of HIV-1 subtypes A, C and D and detected recombinants forms.

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) exhibits extensive genetic variation (Malim & Emerman, 2001). Phylogenetic analyses have resulted in the classification of HIV-1 into three distinct groups: the major (M), outlier (O) and non-M/non-O (N) (Robertson et al., 2000). Group M comprises most HIV-1 infections worldwide and has been subdivided further into nine distinct subtypes (A–D, F–H and J–K) and a number of recombinant viruses classified as circulating recombinant forms (Robertson et al., 2000). So far, there is no clear evidence for subtype-specific variation in virulence or transmission.

HIV-1 mother-to-child transmission rates vary from 15 to 45 % (De Cock et al., 2000). Many factors, such as maternal stage of the disease, maternal immunological status, virus load, mode of delivery, duration of breast-feeding and availability of antiviral therapies, can contribute to these differences (McGowan & Shah, 2000). Nevertheless, the role of virus determinants in mother-to-child transmission has not been well established yet (Dickover et al., 2001). Several studies have shown that maternal diversity is generally higher than that present in the infant (Scarlati et al., 1993), suggesting that maternal viruses are selected before transmission. However, it is still unknown which and how many genetic determinants drive this selection. Variation in the envelope (env) gene, particularly the V3 region, has been shown to correlate with coreceptor affinity and cell tropism, as well as immune evasion. Additionally, it has been suggested recently that the V3 region of env could be a key determinant for mother-to-child transmission (Renjifo et al., 1999). Therefore, V3 seems to be an ideal coding region to identify biological differences in mother-to-child transmission between the different HIV-1 subtypes (Becker-Pergola et al., 2000). Since differences in transcriptional regulation have been observed among different HIV-1 subtypes (Jeeninga et al., 2000; Montano et al., 1997; Rodenburg et al., 2001), in addition to the V3 region, the long terminal repeat (LTR) region has been analysed also in previous studies of mother-to-child transmission of different HIV-1 subtypes (Blackard et al., 2001).

To investigate whether the subtype of HIV-1 may affect HIV-1 mother-to-child transmission rates, we have carried out a sequence analysis of the env (C2V3C3) and LTR (U3)
region of 31 samples from pregnant mothers from Ifakara, a semi-rural area of southeastern Tanzania. The genetic divergence between the different viruses studied was analysed also.

METHODS

Study population and sample collection. The study group involved 980 pregnant women and their 985 infants. Infants were enrolled in an intervention trial carried out in Ifakara (Tanzania) during January to October of 1995 (Menendez et al., 1997, 1999). Maternal blood samples were taken at delivery. Antibodies to HIV were detected in 66 mothers (6-7%) (Menendez et al., 1999). However, only serum samples from 31 children were available for the present study. The children’s blood samples were taken at 5 months of age. Blood samples were collected into EDTA tubes for haematological assessment and serum was aliquotted and stored frozen at −20°C. None of the woman studied had ever received antiretroviral therapy.

Fourteen children tested positive for HIV-1 antibodies by ELISA. However, children were only considered infected when they were positive for HIV-1 RNA or DNA, as assessed using LTR or *env* RT-PCR (see below). Eight children were found to be HIV-1 RNA- or DNA-positive and were consequently considered to be infected with HIV-1. Products from the children’s HIV-1 PCRs were sequenced and the sequences matched with their corresponding mothers (data not shown).

Recovery, amplification and sequencing of viral RNA. RNA was extracted from 140 μl of serum using the RNA Qiam Blood kit (Qiagen) according to the manufacturer’s protocol and stored at −70°C. After viral RNA isolation, 10–20 μl of resuspended RNA (corresponding to 28 μl of serum) were reverse-transcribed at 42°C using the avian myeloblastosis virus RT (Promega). Two different HIV-1 genomic regions were targeted for PCR amplification: the U3 region from the LTR (223 bp) and the C2V3C3 env region (422 bp). Nested PCR was then performed with AmpliTag Gold DNA polymerase and buffers (Perkin Elmer) and under the conditions recommended by the manufacturer. For first-round PCR, 5 μl of the RT product was amplified. LTR primers used for first-round amplification were NI 25 (HXB2 positions 57–77) and NI 23 (HXB2 positions 389–408) (Ibanez et al., 2001). The following amplification conditions were used: 10 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 55°C and 40 s at 72°C, and a final extension step at 72°C for 10 min. LTR primers used for second-round amplification were NI 33 (HXB2 positions 350–372) and NI 35 (HXB2 positions 81–100), as described in Ibanez et al. (2001), following the same conditions to those in the first round. The same reagents and reaction conditions were followed for the amplification of the C2V3C3 region. The outer pair of primers for *env* amplification were env1 (HXB2 positions 6858–6878, 5’-CCAATTCCYA- TACATTATTGT-3’) and env4 (HXB2 positions 7520–7539, 5’-ATGGGAGGGGCATACATTGCT-3’) and the inner primers were env2 (HXB2 positions 6885–6904, 5’-GCTGCTTWTGGGATY- CTAAA-3’) and env3 (HXB2 positions 7365–7385, 5’-TGWATT- RCARTAGAAAAATTC-3’). Both strands of the PCR fragments were sequenced directly using internal (nested) PCR primers and the ABI Prism Dye Terminator Cycle Sequencing Reaction kit (Perkin Elmer). The products of the reactions were then analysed on an ABI 310 sequencer. Sequence editing was performed using the program SEQUENCER, version 4.1 (GeneCodes).

Measurement of virus load. Mother RNA levels are shown in Table 1. Serum HIV-1 RNA levels were measured by the Amplicor Monitor assay (Roche).

Phylogenetic analyses. Sequences were aligned both manually and using the program CLUSTAL W (Thompson et al., 1994). Individual LTR and *env* sequences were classified into HIV-1 subtypes by comparison with control HIV-1 subtype sequences retrieved from the Los Alamos HIV-1 Sequence Database (Kuiken et al., 2000). Alignments were subjected to phylogenetic analyses using programs from the PHYLIP phylogeny inference program (http://evolution.genetics.washington.edu/phylip.html) (Felsenstein, 1988). The neighbour-joining method (Saitou & Nei, 1987) and the Kimura two-parameter model (Kimura, 1980) were applied for calculation of evolutionary distances on the basis of maximum-likelihood distances (programs DNADIST and NEIGHBOR). A transition/transversion ratio of 2 was used. Bootstrap resampling (100 replications) was used to assess the robustness of the phylogenetic trees (programs SEQBOOT and CONSENSE). The phylogenetic tree was plotted using the TREEVIEW program (Page, 1996). Intrasubtype distances were calculated as Hamming distances, or \((1 – s) \times 100\), where ‘s’ is the fraction of shared sites in two aligned sequences, with the program WET (http://www.tdl.es/programas/WET-i.html).

Statistical analyses. To test for differences in the mother-to-child transmissibility of HIV-1 subtypes A, C and D and recombinant forms, we used Fisher’s exact test analysis. Since intrasubtype genetic distances variation was not normally distributed and the studied cohort was relatively small, non-parametric analyses were performed to test for differences between intrasubtype genetic distances. Consequently, the Mann–Whitney test was used to check whether the genetic means were significantly different. The Mann–Whitney test was also used to search for significant differences between the virus loads of transmitters and non-transmitters. *P* values of less than 0-05 were considered statistically significant. Analyses were performed using the spss software package, version 10.0.

RESULTS

Analysis of virus load

The mean level of HIV-1 RNA (copies ml⁻¹ of plasma) in transmitters was not significantly higher than that in non-transmitters \([19 \, 223 \pm 32 \, 661 \text{ (range } 1090–98 \, 900\text{), median } 6850, \text{ versus } 44 \, 529 \pm 103 \, 601 \text{ (range } 1670–512 \, 000\text{), median } 20 \, 100; \text{ } P=0.132\text{, Mann–Whitney } U \text{ test}]. Similarly, no significant differences were found when the mean virus loads of different subtypes were compared \([27 \, 850 \pm 20 \, 856 \text{ (range } 11 \, 900–62 \, 300\text{), median } 18 \, 200; \text{ } 54 \, 976 \pm 138 \, 470 \text{ (range } 2270–512 \, 000\text{), median } 13 \, 300; \text{ } 35 \, 288 \pm 32 \, 809 \text{ (range } 1090–98 \, 900\text{), median } 27 \, 000; \text{ and } 13 \, 820 \pm 13 \, 406 \text{ (range } 4340–23 \, 300\text{), median } 13 \, 820; \text{ for subtypes } C, A, \text{ recombinant and } D, \text{ respectively} \) (Table 1).

LTR and envelope subtyping

HIV-1 genetic subtyping of the 31 Tanzanian isolates was performed by direct sequencing of the U3 LTR and C2V3C3 env regions (Table 1). To determine the LTR and envelope subtype of these 31 isolates, phylogenetic trees with the study samples and the Los Alamos subtype reference sequences were constructed (Figs 1 and 2). Thirteen samples (3, 7, 8, 10, 12, 13, 15, 16, 18, 25, 26, 28 and 30) were classified as subtype A, six (1, 5, 14, 23 and 29) as subtype C and two as subtype D (9 and 19) (Table 1). Although no amplification of the env region was achieved in three
Transmission of different HIV-1 subtypes

samples (21, 22 and 24), they were subtyped in the LTR region as C (21 and 22) and A (24). Finally, seven samples presented a different subtype in LTR and envelope (2 A/C, 4 C/D, 11 C/A, 17 A/C, 20 D/A, 27 A/C and 31 C/A) (Table 1). In short, among the 28 samples amplified in both regions, we have found that 13 (46 %) of the samples were subtype A, six (21 %) belong to subtype C, two (7 %) were subtype D and seven (25 %) were recombinants (Tables 1 and 2).

Rate of transmission of different subtypes

Of the above 31 HIV-infected mothers, eight had infected their infants (26 %). Among these eight transmitters, we identified four with subtype A (4 of 13, 31 %), one with subtype C (1 of 6, 17 %), none with subtype D (0 of 3, 0 %) (Tables 1 and 2). Similar to the previous analysis, no significant differences were found (A/C, P = 0.325; A/D, P = 0.446; C/D, P = 0.255; Fisher’s exact test). When the LTR region was analysed, we identified seven transmitters with subtype A (7 of 17, 41 %), one with subtype C (1 of 11, 9 %) and none with subtype D (0 of 3, 0 %). Again, no significant differences were identified (A/C, P = 0.100; A/D, P = 0.251; C/D, P = 0.769; Fisher’s exact test). These results showed a similar transmission rate of the three subtypes analysed in this study.

Genetic divergence between subtypes

To assess the intrasubtype genetic diversity, genetic distances between the sequences of each clade were calculated. The mean intrasubtype LTR genetic distances observed among A, C and D sequences were 9 % (range 4–17 %), 14 % (range 5–25 %) and 14 % (range 13–15 %), respectively (Fig. 3). The observed C2V3C3 env mean intrasubtype genetic distances were 13 % (range 2–22 %), 13 % (range

Table 1. HIV-1 subtype and virus load of transmitter and non-transmitter mothers from the Ifakara (Tanzania) cohort

Infected children were detected by amplifying HIV-1 RNA or DNA, using LTR or env RT-PCR.

<table>
<thead>
<tr>
<th>Transmitter mothers</th>
<th>Non-transmitter mothers</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>LTR subtype</td>
</tr>
<tr>
<td>-----</td>
<td>-------------</td>
</tr>
<tr>
<td>1</td>
<td>C</td>
</tr>
<tr>
<td>2</td>
<td>A</td>
</tr>
<tr>
<td>3</td>
<td>A</td>
</tr>
<tr>
<td>13</td>
<td>A</td>
</tr>
<tr>
<td>17</td>
<td>A</td>
</tr>
<tr>
<td>18</td>
<td>A</td>
</tr>
<tr>
<td>27</td>
<td>A</td>
</tr>
<tr>
<td>30</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>D</td>
</tr>
<tr>
<td>22</td>
<td>C</td>
</tr>
<tr>
<td>24</td>
<td>A</td>
</tr>
<tr>
<td>26</td>
<td>A</td>
</tr>
<tr>
<td>28</td>
<td>A</td>
</tr>
<tr>
<td>30</td>
<td>A</td>
</tr>
</tbody>
</table>

*Recombinant sequences that present different subtype between LTR and env sequences.
Fig. 1. Neighbour-joining phylogenetic reconstruction of HIV-1 LTR region nucleotide sequences obtained from serum samples of the mothers analysed in this study. The PHYLIP program was used to construct the trees by means of a Kimura distance matrix. Numbers at branch nodes refer to bootstrap proportions 100 bootstrap replications. Subtype determination was based on tree analysis using reference sequences of known HIV-1 env subtypes (Kuiken et al., 2000) (http://hiv-web.lanl.gov). The HXB2 strain was used as an outgroup.
6–22 %) and 21 % (range 17–27 %) for subtype A, C and D, respectively. Therefore, subtype D was the one with more divergent C2V3C3 env sequences, followed by subtypes C and A. (Fig. 3). The mean LTR intrasubtype genetic variation was significantly different between each subtype \((P > 0.0001, \text{Mann–Whitney test})\), except between subtypes C and D. Likewise, we also calculated the C2V3C3 intrasubtype genetic variation, which was statistically significant between subtypes A and D \((P > 0.0001)\) but not between subtypes C and D or A and C. Therefore, the intrasubtype genetic distances observed in the Ifakara (Tanzania) cohort were relatively high.

Fig. 2. Neighbour-joining phylogenetic reconstruction of HIV-1 env (C2V3C3) region nucleotide sequences obtained from serum samples of the mothers analysed in this study. Analyses were carried out as described in Fig. 1.
DISCUSSION

Three different HIV-1 subtypes, A, C and D, have been identified in Tanzania (Hoelscher et al., 1998, 2001). The sequences presented here, sampled in 1995, were subtypes A (46 %), C (21 %), D (7 %) and recombinants (25 %). This result is consistent with sequence data from other studies in which southern Tanzanian HIV-1 isolates were characterized (Hoelscher et al., 2001).

The present study was designed to assess whether LTR and envelope HIV-1 subtypes could affect the mother-to-child transmission rate. This transmission rate is similar to that reported in recent clinical trials in Africa (Eshleman et al., 2001). Regarding the influence of the HIV-1 subtype in the rate of mother-to-child transmission, our results suggest that there is no difference in the relative rate of transmission between subtypes A, C or D. However, the low number of mothers infected with subtype D limits the power of the study. Similar results have been documented from other sub-Saharan countries, where the major strains seem to be A, C and D (Becker-Pergola et al., 2000; Murray et al., 2000). In contrast, a study from Tanzania suggested that subtype D is less likely to be transmitted mother-to-child than subtypes A, C or intersubtype recombinants (Renjifo et al., 1999, 2001). The former study, based on the analysis of the env V3 region, also suggested that the fitness of subtype D V3 might be reduced in mother-to-child HIV-1 transmission.

Since subtype-specific differences among LTRs have been observed (Jeeninga et al., 2000; Naghavi et al., 1999) and the LTR subtype has been also associated with different rates of mother-to-child transmission (Blackard et al., 2001), the LTR region of the 31 HIV-1-infected mothers included in the present study was also analysed. However, the LTR subtype did not seem to influence the rate of mother-to-child transmission in this study. It has to be mentioned that other variables, such as maternal virus load or maternal immunological status, may affect the rate of mother-to-child transmission. Nevertheless, no significant differences were observed in the present study between the virus loads of transmitter and non-transmitter mothers (Table 1).

An interesting finding is the high intrasubtype genetic diversity observed among viruses cocirculating in a relative small village, Ifakara, located in southeastern Tanzania. In particular, the high genetic diversity found within subtypes C and D is remarkable. The degree of intrasubtype diversity is equivalent to that reported when comparing viruses isolated from different sub-Saharan countries (Vidal et al., 2000). This result may be informative of the rapid spread of different genotypes through southeastern Africa, as it seems to be the case for subtype C which is increasing in prevalence in this geographical area (Renjifo et al., 1999). However, it has also been suggested for other sub-Saharan countries that this high intrasubtype genetic diversity can be also due to an old epidemic of these subtypes in this particular geographical area (Vidal et al., 2000). In any case, the high intrasubtype genetic diversity observed here in a very localized geographical area, together with the increasing prevalence of intersubtype recombinants, denotes the difficulty for future vaccine development as well as for an efficient antiretroviral treatment.

Table 2. HIV-1 subtype prevalence and rate of mother-to-child transmission in the Ifakara (Tanzania) cohort

<table>
<thead>
<tr>
<th>Subtype</th>
<th>HIV-1 subtype prevalence (%)</th>
<th>Rate of transmission (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LTR</td>
<td>env</td>
</tr>
<tr>
<td>A</td>
<td>17 (54)</td>
<td>16 (57)</td>
</tr>
<tr>
<td>C</td>
<td>11 (35)</td>
<td>9 (32)</td>
</tr>
<tr>
<td>D</td>
<td>3 (10)</td>
<td>3 (11)</td>
</tr>
<tr>
<td>Recombinants</td>
<td>7 (25)</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3. Distribution of the genetic distances in the LTR (U3) and env (C2V3C3) regions. Horizontal lines indicate the median nucleotide distances. Interquartile ranges and minimum and maximum distances are indicated by boxes and bars, respectively.
ACKNOWLEDGEMENTS

Work at Fundació irsiCaixa was supported by grants from Fundació irsiCaixa and Fondo de Investigaciones Sanitarias (FIS2001-0067-02). Work at Hospital Clinic was supported by Generalitat de Catalunya and Spanish Agency for International Cooperation and UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases. The Ifakara Center receive major core funding from the Swiss Agency for Development and Cooperation. Financial support to N. Tapiá was granted by FIS, to S. Franco by Fundació Clinic and to F. Puig-Basagoiti by BEFI-9013/99-FIS. We thank the parents and guardians of all the children in this study. We are also grateful to the staff of the Ifakara Center.

REFERENCES


