Human Immunodeficiency Virus Type 1 Subtype F Reverse Transcriptase Sequence and Drug Susceptibility

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We sequenced and phylogenetically analyzed the reverse transcriptase (RT) regions of the pol genes of 14 human immunodeficiency virus type 1 (HIV-1) isolates from Romanian patients, which were classified as subtype F on the basis of env gene structure. The RT sequences showed that the strains clustered phylogenetically and were equidistant from other HIV-1 subtypes as shown by the neighbor-joining and maximum-likelihood methods, allowing us to define HIV-1 subtype F according to the pol classification. The subtype F RT sequences differed from reported group M RT sequences by 10.94% (for nucleotides) and 7.6% (for amino acids). Phenotypic analysis of subtype F susceptibility to three classes of antiretroviral compounds showed an increase in the 50% inhibitory concentration of the tetrahydroimidazo[4,5,1-jk][1,4]-benzodiazepin-2-(1H)-one and -thione (TIBO) derivative R82913 for one strain which was naturally resistant to this compound. This first report of subtype F pol sequences confirms the perfect correlation between the phylogenetic positions determined by env and pol analyses and suggests that virus variability might influence the efficacy of antiretroviral treatments. This finding warrants a global evaluation of the phenotypic and genotypic susceptibility of HIV-1 subtypes to antiretroviral drugs.

Most human immunodeficiency virus type 1 (HIV-1) drug susceptibility studies have involved subtype B. Little information on the impact of viral diversity on natural susceptibility to antiretroviral drugs has been reported to date. However, HIV-1 group O viruses are naturally resistant to nonnucleoside reverse transcriptase (RT) inhibitors (8), as is HIV-2 (28). Subtypes are defined on the basis of the env (24, 25) or gag (19) gene. Most RT sequences reported to date belong to subtype B strains, which prevail in North America and Western Europe (25), i.e., regions where antiretroviral drugs are developed and clinical trials are conducted. RT sequences of subtypes A to D are also available (25, 32, 34). The full sequences of Thai strains defined as subtype E according to env classification and defined as subtype A on the basis of the gag gene (19) also corresponded to subtype A on the basis of the pol gene (5, 14). By contrast, full sequence analysis of a subtype G strain ruled out recombination events in the pol gene (6), in keeping with a previous report on the RT sequences of subtype G (17).

We present the first RT gene sequences and data on the phenotypic susceptibility to antiretroviral drugs of subtype F strains from Romanian patients. This is the dominant subtype in Romanian children and adults (1, 11) and is also a minor viral form in other countries, such as Brazil (23), Argentina (4), Cameroon (27), Russia (18), Taiwan (7), Martinique (10), Cyprus (16), France (33), Belgium (13), and The Netherlands (20). Recombinant F/B strains have also been reported (21, 30).

MATERIALS AND METHODS

Study population. We studied 14 HIV-1 subtype F strains isolated from Romanian children (n = 9) and adults (n = 5). All but one of the infected children were nosocomially infected (by injections with nonsterile, reused needles and syringes); the remaining one was vertically infected. Clinical and epidemiological data are described elsewhere, together with virus isolation, env subtype determination, and strain codification (1). None of the patients had received antiretroviral therapy.

HIV-1 RT sequencing. DNA was extracted with phenol-chloroform from cocultured peripheral blood mononuclear cells from (PBMC) infected patients, precipitated with ethanol, and quantified spectrophotometrically. The env gene was then amplified in a nested PCR with outer primers RT-18 and RT out and inner primers RT-19 and RT-20 as previously described (26). Each nested-PCR product (1,008 bp) was subjected to direct population sequencing with sense primer AE5 (5'-ATTCTCCATATTGCCTATT-3') and antisense primer NE1 (5'-ATGTCATTGACGTCAGCTG-3'). Sequencing reactions were run with the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq DNA polymerase (F. Perkin-Elmer) on an automated sequencer (Applied Biosystems 373A).

Phylogenetic analysis. DNA sequences were analyzed with the multiple-sequence editor Clustal W (35) and improved by visual inspection. The sequences were gapstripped and a pairwise matrix based on 591 sites was generated with the DNADIST program of the PHYLIP package, version 3.56 (12). Tree topology was inferred by the neighbor-joining method with the Kimura two-parameter distance matrix (PHYLIP) and a transition/transversion ratio of 2. Bootstrap analysis was performed with the SEQBOOT (100 resamplings), DNADIST, NEIGHBOR, and CONSENSE programs (PHYLIP package). Phylogenetic analysis was also performed by the maximum-likelihood method, using the DNASP program (12). The tree outlied the HIV-1 group O sequences (MVP5180 and ANT70). In the tree construction we also included the RT sequences of six subtype B HIV-1 isolates (LAI, SF2, MN, OYI, IRFL, and JRCSF), three subtype D sequences (ELI, NDK, and ZZ36), two subtype A sequences (UG037 and U455), the sequence of an A/G recombinant strain (HRNG) (14), and the sequence of a presumed A/D recombinant strain (MAL) from the Los Alamos National Laboratory database (25). The newly reported sequences for strains CM240 (5), 90CR402, and 93TH253 (14), recombinant A/B strains, were also used in the phylogenetic analyses.

Phenotypic susceptibility assay. The phenotypic susceptibilities of the cellular HIV-1 subtype F isolates were analyzed in a PBMC assay, taking into account the replication kinetics of each strain (3). After conventional isolation of HIV from frozen PBMC, the cell-free HIV-1 subtype F supernatants corresponding to peak RT activity were serially diluted (10−2 to 10−6) and incubated with fresh normal phytohemagglutinin-stimulated PBMC. After being washed, the infected cells were placed in 96-well plates containing six serial dilutions of the antiretroviral
Each dilution was tested in triplicate. On days 5, 7, and 10, the supernatants were collected and half the medium was replaced with fresh drug-containing medium. The 50% tissue culture infective dose was assessed by measuring RT activity in control drug-free supernatants collected on the same days. At the peak of RT activity we calculated the drug concentrations inhibiting 50 and 90% (IC50 and IC90, respectively) of the RT activity of 100 50% tissue culture infective doses. For zidovudine (ZDV), an IC50 cutoff of 0.05 μM has been defined to classify the virus isolates as ZDV sensitive or ZDV resistant, based on phenotypic analyses of several isolates from treated and untreated patients and on comparisons of these results to genotypic data. For the other antiretroviral drugs, it has not been possible to determine any cutoff value. In this study, phenotypic resistance to these compounds was defined as at least a fivefold increase in IC50s for the HIV-1 subtype F isolates compared to those for HIV-1 subtype B strains.

Antiretroviral agents. We tested the nucleoside RT inhibitors ZDV (Wellcome, Dartford, United Kingdom) and lamivudine (3TC; Glaxo-Wellcome, Dartford, United Kingdom); the nonnucleoside RT inhibitors tetrahydroimidazo[4,5-j]-1,4-benzodiazepin-2(1H)-one and -thione (TIBO) derivate R82913 (Janssen, Beerse, Belgium), delavirdine (DLV; Upjohn, Kalamazoo, Mich.), and nevirapine (NVP; Boehringer Ingelheim Pharmaceuticals, Ridgefield, Conn.); and the protease inhibitors saquinavir (SQV; Roche, Welwyn Garden City, United Kingdom) and ritonavir (RTV; Abbott, Abbott Park, Ill.). The purified drugs were kindly provided by the manufacturers.

Nucleotide sequence accession numbers. The nucleotide and amino acid sequences of codons 33 to 235 of the RT genes and proteins from the 14 HIV-1 subtype F isolates have been submitted to GenBank (accession no. Y16138 to Y16151).

RESULTS

Genetic analysis. Figure 1 shows the amino acid alignment of codons 33 to 235 of the RT genes of the different subtype F isolates. The subtype F consensus sequence differed from that of subtype B in 11 residues (V32T, I37A, E40D, D131E, I135L, S162Y, M173I, Q174K, V178I, Q207A, and R211K). Two of these mutations (A39 and E131) are frequently present in subtype F strains, and none of the sequences analyzed contained mutations previously linked to resistance to nucleoside or non-nucleoside RT inhibitors. The sequence of the RO-BCI23 isolate was more similar to that of the subtype B consensus sequence in the first part of the RT gene (subtype B structure in sequences encoding T39, E40, and I135) but was more variable than the other subtype F isolates in the second half of the RT gene (close to the binding pocket). Compared to the subtype B consensus sequence, the RO-BCI23 protein bore four mutations close to the active site of the RT protein (E169D, K173A, Q174K, and D177E).

At position 60, all but one of the strains originating in nosocomially infected Romanian children (RO-BCI7, RO-BCI8, RO-BCI9, RO-BCI11, RO-BCI12, RO-BCI15, and RO-BCI16) bore a valine (as in the subtype B consensus sequence), whereas the strains isolated from adults (RO-BCI17, RO-BCI18, RO-BCI19, RO-BCI20, and RO-BCI23), a vertically infected child (RO-BCI13), and a nosocomially infected child from whom the strain was isolated in 1994 (RO-BCI1) bore an isoleucine. Conversely, at position 39, all but one (RO-BCI17) of the strains isolated from adults bore a threonine, like subtype B strains, whereas all those isolated from nosocomially infected children bore an alanine.

Phylogenetic analysis of HIV-1 RT sequences. Phylogenetic analysis of the RT nucleotide sequences by the neighbor-joining and maximum-likelihood methods gave similar results.
Phylogenetic trees were constructed by using the 14 Romanian HIV-1 RT sequences, representative RT sequences of subtype B strains, and sequences belonging to different subtypes obtained from the database (25). Figure 2 shows the phylogenetic tree constructed by neighbor joining. High bootstrap values were obtained at the relevant nodes, indicating that subtypes B, D, and F each form a consistent clade. Subtype A was composed of five different strains defined as A or E on the basis of the env classification, whereas recombinant strains IBNG and MAL were subtype outliers.

The subtype F phylogenetic tree showed that the strains from nosocomially infected Romanian children formed a clade, as was previously observed by analyzing the env gene (1). Strain RO-BCI13 (+) was isolated from a vertically infected child. Strains RO-BCI17, RO-BCI18, RO-BCI19, RO-BCI20, and RO-BCI23 were isolated from HIV-1-infected adults.

**Phenotypic susceptibility.** The IC_{50}s of nucleoside analogs ZDV and 3TC were similar to those for wild-type subtype B field isolates. Although the IC_{50}s were higher for the subtype F strains than for the subtype B strains, all the subtype F isolates were susceptible to protease inhibitors. All were also sensitive to the nonnucleoside RT inhibitors NVP and DLV. Susceptibility to the third nonnucleoside RT inhibitor, TIBO derivate R82913, was lower for two isolates (Table 2); one isolate (RO-BCI11) showed borderline susceptibility, with a moderate increase in IC_{50} and IC_{90} (0.30 and 1.1 μM, respectively), the second strain (RO-BCI23) showed a significant increase in both IC_{50} and IC_{90} (0.53 and 2.02 μM, respectively). This phenotype was not associated with any of the known mutations linked to TIBO resistance. However, the RO-BCI23 isolate showed the most variable RT sequence of the subtype F isolates and presented different residues close to the active site of the RT. We therefore assessed the phenotypic susceptibilities of all the available subtype F isolates to TIBO. The TIBO IC_{50} (mean ± standard deviation) for 12 subtype F isolates was 0.07 ± 0.06 μM (range, 0.01 to 0.2 μM), and the IC_{90} was 0.4 ± 0.27 μM (range, 0.01 to 0.88 μM), meaning that all 12 strains were susceptible.

**DISCUSSION**

Studies of HIV’s genetic diversity have shown different rates of variability for the different viral genes, the most conserved structural gene being pol (29, 31). Analysis of the pol gene was not considered relevant for genotyping (36), but several recent reports have shown that the numerous selection pressures on the pol gene, generally reflected by synonymous substitutions, make it suitable for phylogenetic studies (32, 34). The different pol subtypes described so far correspond to env or gag subtypes (18, 25, 32, 34).

We analyzed the RT coding regions of 14 isolates originating in different parts of Romania and characterized as subtype F on the basis of the env sequence (1). Based on a pol nucleotide and amino acid sequence comparison, these strains clustered phylogenetically and were equidistant from other HIV-1 subtypes. This first report of subtype F pol sequences confirms the perfect correlation between the phylogenetic positions determined by env and gag analysis. The similar results obtained by the neighbor-joining and maximum-likelihood methods support the reliability of the phylogenetic tree of HIV-1 RT sequences. Furthermore, phylogenetic analysis of the pol gene of Romanian HIV-1 isolates revealed a cluster similar to that obtained by analyzing the env genes sequences. The sequences of isolates from nosocomially infected children formed a separate branch within the Romanian sequence cluster with a high

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**TABLE 1. Nucleotide and amino acid divergences between subtype F and other group M subtype and group O RT sequences**

<table>
<thead>
<tr>
<th>Isolate group</th>
<th>% Divergence from subtype F (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nucleotides</td>
</tr>
<tr>
<td>Group M</td>
<td>10.94 (8.04-14.84)</td>
</tr>
<tr>
<td>Subtype B</td>
<td>10.04 (8.04-13.15)</td>
</tr>
<tr>
<td>Subtype A</td>
<td>11.08 (9.35-14.84)</td>
</tr>
<tr>
<td>Subtype D</td>
<td>11.01 (9.55-11.96)</td>
</tr>
<tr>
<td>Group O</td>
<td>32.09 (29.9-33.52)</td>
</tr>
</tbody>
</table>

*Average intrasubtype F divergence was 3.56% (range, 1.54 to 6.36%) for nucleotides and 3.7% (range, 1.57 to 6.7%) for amino acids.
performed on 11 HIV-1 group O isolates as previously described 9.

IC50s and IC90s were measured for isolates from untreated patients a.

TABLE 2. IC50s and IC90s of nucleoside and nonnucleoside RT inhibitors and protease inhibitors of HIV-1 subtype F isolates, reference HIV-1 isolates, and HIV-2

<table>
<thead>
<tr>
<th>Isolate</th>
<th>ZDV (nM)</th>
<th>3TC (nM)</th>
<th>DLV (nM)</th>
<th>NVP (nM)</th>
<th>TIBO (nM)</th>
<th>RTV (nM)</th>
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</thead>
<tbody>
<tr>
<td>RO-BC1</td>
<td>&lt;0.002</td>
<td>0.03</td>
<td>0.03</td>
<td>0.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RO-BC15</td>
<td>&lt;0.002</td>
<td>0.03</td>
<td>0.03</td>
<td>0.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RO-BC16</td>
<td>&lt;0.002</td>
<td>0.03</td>
<td>0.03</td>
<td>0.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RO-BC17</td>
<td>&lt;0.002</td>
<td>0.03</td>
<td>0.03</td>
<td>0.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RO-BC18</td>
<td>&lt;0.002</td>
<td>0.03</td>
<td>0.03</td>
<td>0.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RO-BC19</td>
<td>&lt;0.002</td>
<td>0.03</td>
<td>0.03</td>
<td>0.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RO-BC23</td>
<td>&lt;0.002</td>
<td>0.03</td>
<td>0.03</td>
<td>0.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RO-BC24</td>
<td>&lt;0.002</td>
<td>0.03</td>
<td>0.03</td>
<td>0.16</td>
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<td></td>
</tr>
<tr>
<td>RO-BC25</td>
<td>&lt;0.002</td>
<td>0.03</td>
<td>0.03</td>
<td>0.16</td>
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