Coinfection and Superinfection in Patients with Long-Term, Nonprogressive HIV-1 Disease

Concepción Casado, 1 María Pernas, 1 Tamara Alvaro, Virginia Sandoonis, 1 Soledad García, 2 Carmen Rodríguez, 2 Jorge del Romero, 2 Eulalia Grau, 2 Lidia Ruiz, 2 and Cecilio López-Galindez 3

1Centro Nacional de Microbiología Instituto de Salud Carlos III, and 2Centro Sanitario Sandoval, Instituto Madrileño de la Salud, Comunidad Autónoma de Madrid, Madrid, and IrsiCaixa Foundation, Hospital Germans Trias i Pujol, Barcelona, Spain

Human immunodeficiency virus 1 (HIV-1) dual infections are considered important because they have been related to AIDS progression. We identified dual infections in 2 patients with long-term, nonprogressive HIV-1 disease; the first patient was diagnosed as being already coinfected, on the basis of the first sample analyzed, but a previous superinfection could not be excluded; the second patient was diagnosed as having a superinfection, on the basis of the 9-year difference between the viral dating of the 2 strains. Dual infections occur in patients with long-term, nonprogressive disease, with no immediate clinical manifestations. Such occurrences could indicate a general phenomenon in natural HIV-1 infections.

Infection with more than 1 strain of HIV-1 has been considered to be an extraordinary event in the natural history of the infection. However, the detection of dual infections in patients with HIV-1, as either a coinfection or a superinfection, has been described, mostly in individuals who have engaged in high-risk practices [1–6]. Obtaining systematic data on the frequency of HIV dual infections is difficult, but, when different subtypes circulate, the identification of different strains becomes feasible, and superinfection seems to be more frequent than previously thought [7, 8]. Moreover, the detection of many circulating recombinant forms and unique recombinant forms [8] argues that it occurs at high frequency in the natural history of HIV-1.

The importance of HIV-1 dual infections is related to their pathogenic consequences, because most studies have found an elevated HIV-load set point and disease progression after superinfection [3, 5, 9]. However, the clinical outcome of HIV-1 dual infection needs to be definitively established. Here we report the identification of dual infections without apparent clinical consequences in 2 patients infected, for >18 years, with long-term, nonprogressive (LTNP) HIV-1 disease.

Patients and methods. Patient 1 was a former intravenous drug user followed up in an outpatient clinical center (Centro Sanitario Sandoval, IMSALUD), and patient 2 was a homosexual man treated in the HIV Clinical Unit (Hospital Germans Trias i Pujol, Laboratory of Retrovirology, IrsiCaixa Foundation). Both patients were included in the study after they gave free and informed consent, according to the guidelines of the institutional ethical committees. Patient 1 had been infected for at least 18 years, patient 2 for 20 years. Both patients had remained asymptomatic without antiretroviral therapy and with CD4 levels >500 cells/mL, fulfilling the LTNP criterion. The immunologic and virologic data, together with the samples analyzed, are summarized in figure 1. Plasma HIV-1 RNA was quantified by use of the Amplicor HIV Monitor test kit, with a detection limit of 50 copies/mL (Roche Diagnostics Systems), according to the manufacturer’s instructions.

Five samples from each patient were analyzed (figure 1). Peripheral blood mononuclear cells were obtained by use of standard protocols, and viral DNA was obtained from 1 × 10^7 cells by a standard phenol-extraction method. HIV DNA was amplified from a single copy in the C2–V5 region of the env gene, by use of limiting-dilution polymerase chain reaction (PCR) with different primers [10]. The final PCR products (661 bp), spanning the C2–V5 region of the HIV env gene, were sequenced by use of primer 27EU and the ABI PRISM Dye Terminator reaction kit (Perkin-Elmer), according to the manufacturer’s instructions, in an ABI PRISM 377 automated sequencer.

Unique nucleotide sequences obtained from different points in each patient were aligned by use of the CLUSTAL X program together with HIV reference sequences (http://www.hiv.lanl.gov/content/index) and with Spanish-patient nucleotide sequences, obtained from the HIV database and from our laboratory, and then were hand-edited. All positions with an align-
ment gap were excluded from the analysis. Best-fit models of nucleotide substitution were selected according to the Akaike information criterion in Modeltest (version 3.6; David Posada Web page, http://www.darwin.uvigo.es). Maximum-likelihood trees were estimated under the best-fit model by use of the algorithm implemented in Phylm (version 2.4.1; http://www.atge.lirmm-fr/phylm) and by starting the search from a BIONJ tree. Phylogenetic confidence was assessed by bootstrap analysis of 1000 replicates \[10\]. Identical sequences derived from the same or different samples were used only once to reconstruct the phylogenetic tree shown in figure 2.

To perform the viral dating of the different viral populations in each patient, the genetic distance of the reconstructed most recent common ancestor (MRCA) for each clade was compared with that of a reconstructed MRCA for the Spanish epidemic. The viral dating time was estimated by use of a linear-correlation equation previously developed on the basis of a large set of Spanish samples, an equation that correlates the V3 nucleotide-sequence divergence to the Spanish-epidemic MRCA and the sampling time \[11, 12\]. (Genbank accession numbers are EF517970–EF518214.)

**Results.** Phylogenetic analysis of viral sequences obtained from Spanish patients with LTNP HIV-1 disease and of nucleotide sequences derived from unrelated Spanish patients with HIV-1 disease (either LTNP or typically progressive) showed that all isolates were subtype B. Nucleotide sequences obtained from each patient formed monophyletic groups with high bootstrap values (>75%), except for patients 1 and 2, each of whose nucleotide sequences were separated into 2 groups (a and b in figure 2), situated in different branches of the tree, and supported by bootstrap values >85%.

The mean genetic distances between groups a and b—5.4% for patient 1 and 11.2% for patient 2 (table 1)—were higher than the genetic distances to other nonepidemiologically related Spanish HIV-1 isolates (table 1), as indicated in other studies \[3\]. For patient 1, the range of genetic distances between each clade and unrelated HIV Spanish isolates was 2.2%–17.8% for clade a and 2.0%–18.3% for clade b; the corresponding values for patient 2 were 6.3%–19.2% for clade a and 7.4%–20.4% for clade b. The presence of 2 statistically differentiated clusters within these 2 patients’ quasispecies, together with the greater genetic distance between clusters a and b compared with that in unrelated viruses, indicates that HIV dual infection with subtype B has occurred in these 2 patients.

Because the 2 groups (i.e., a and b) of nucleotide sequences were present in all the analyzed samples from each patient and because no samples close to the seroconversion time were available, it was not possible to determine whether coinfections or superinfections had occurred. To address this problem, we performed viral dating of the different patients’ clusters, according to the method that our laboratory had established for Spanish isolates and that has been explained in the “Patients and methods” section above. According to this method, we interpolated the year of the nucleotide sequence of each of the different patient clades (table 1). The years obtained for clades a and b for patient 1 were identical (i.e., 1992), whereas those obtained for clades a and b for patient 2 were different; for the latter patient, the dating year for clade a was 1987, close to the seroconversion time, and the dating year for cluster b was 1996 (i.e., 9 years later). The viral dating indicates that a superinfection had occurred in patient 2, whereas analysis of the first sample from patient 1 showed that he already wascoinfected, although a previous superinfection could not be ruled out.

**Discussion.** In the present report, we have presented data on dual infections, with subtype B variants, in 2 patients with LTNP HIV-1 disease who had no clinical manifestations. Although both patients had LTNP characteristics, >15 years of infection, and no clinical symptoms and had not received antiretroviral therapy, they showed different levels of CD4+ T cell counts, RNA viral load, and risk practices. Whereas patient 1 showed multiple peaks in viral load and a continuous but slow decline in CD4+ T cell count, patient 2 showed a more constant...
Figure 2. Maximum-likelihood tree calculated on the basis of unique nucleotide sequences derived from the V3–C5 \textit{env} region from Spanish patients with HIV-1. Nucleotide sequences from patients with long-term, nonprogressive (LTNP) disease are underlined, except for those from patient 1 (white circles, denoted by boldface “1a” and “1b”) and patient 2 (black dots, denoted by boldface “2a” and “2b”). Nucleotide sequences from Spanish patients without LTNP disease are in standard typeface; reference strains from different subtypes are shown in gray and are italicized. Subtrees obtained from LTNP quasispecies analysis are collapsed and are represented by the gray-shaded leftward-pointing triangles. Bootstrap values for each clade are denoted by boldface numbers. All quasispecies studied resulted in single clusters, except for those from patients 1 and 2, which formed 2 different clusters. The scale bar at the bottom of the figure represents 10% genetic distance.

CD4+ T cell count, undetectable or low RNA 1 load, and 2 sporadic blips. Both individuals were and remain classified as having LTNP disease.

We used a phylogenetic approach to distinguish between coinfection and superinfection events. It is known that a molecular clock operates in HIV-1 evolution, and this has permitted estimation of the time of origin of either the global epidemic or the epidemic in individual countries [13], although the approach depends on the method used. Along this line, we have previously demonstrated that a molecular clock operates in the Spanish HIV-1 epidemic [12]. This approach allowed us to estimate the viral dates for different isolates and to detect
sequences with different dates within individual quasispecies [11, 14]. Using this methodological approach, we obtained the same date for the 2 viruses in patient 1 (1992) and different dates (1987 for clade a and 1996 for clade b) for the 2 viruses in patient 2. These results strongly suggest that patient 2 was infected with 1 virus and then, 9 years later, became superinfected with another virus, which showed an 11.2% genetic distance from the first virus. In contrast, patient 1 was coinfected, for at least 18 years, with 2 viruses differing by a genetic distance of 5.4%, although it is not possible to rule out a previous superinfection (table 1). The present study indicates the usefulness of a viral dating strategy to clarify situations that occur in HIV-1 viral evolution in infected patients [11, 14]. These data show that in these 2 patients there was control of the replication of not only a single virus but also of a second, divergent strain. Moreover, the superinfection in patient 2 occurred 9 years after the primoinfection and in the presence of an effective immune control of viral replication.

In most previous reports in the literature, dual infection has been associated with a clinical deterioration of patients [5]. Only 1 case of superinfection in a long-term survivor has been described, and this individual showed a clear progression of the disease after an acute febrile illness and the detection of a superinfecting strain [15]. To our knowledge, the present report is the first to describe the occurrence of dual infection in 2 patients with LTNP HIV-1 disease who have maintained this clinical characteristic and who have not shown any sign of either clinical deterioration or faster clinical progression. Therefore, patients with LTNP disease who have not experienced disease progression for >10 years could be expected, a priori, to have no superinfections—or at least a low rate of superinfection. Moreover, if superinfection occurs in 2 groups of patients with HIV-1 with different characteristics (e.g., typical and LTNP), it can be considered a general phenomenon that probably occurs in every group of infected patients. In summary, in the present study we have found that dual infections also occur in patients with LTNP HIV-1 disease and are not associated with clinical manifestations.

Acknowledgments

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References


Table 1. Characteristics of patients and clades defined in figure 2.

<table>
<thead>
<tr>
<th>Source</th>
<th>First documented HIV-1 test</th>
<th>Year</th>
<th>Heterogeneity within clade, mean ± SE</th>
<th>Genetic distance, % Between clades, % From unrelated isolates</th>
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<tbody>
<tr>
<td>Patient 1</td>
<td>1986</td>
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<tr>
<td>Group a</td>
<td>1992</td>
<td>1.18 ± 0.18</td>
<td>5.4</td>
<td>2.2–17.9</td>
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<tr>
<td>Group b</td>
<td>1992</td>
<td>4.70 ± 0.50</td>
<td>2.0–18.3</td>
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<tr>
<td>Patient 2</td>
<td>1988</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group a</td>
<td>1987</td>
<td>0.93 ± 0.22</td>
<td>11.2</td>
<td>6.3–19.2</td>
</tr>
<tr>
<td>Group b</td>
<td>1996</td>
<td>1.15 ± 0.34</td>
<td>7.4–20.4</td>
<td></td>
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</tbody>
</table>

*Estimated on the basis of the genetic distance to a reconstructed Spanish most recent common ancestor (MRCA), according to the method of Bello et al. [11].
*Calculated in terms of nucleotides and between all pairwise comparisons of sequences within each clade.
*Between the reconstructed most common recent ancestors in the clades.
*Between the reconstructed most recent common ancestor in each clade and the nucleotide sequences obtained from Spanish patients with HIV-1.


