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Prevalence of X4 tropic viruses in patients recently infected with HIV-1 and lack of association with transmission of drug resistance

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***Background*: HIV-1 co-receptor usage may play a critical role in AIDS pathogenesis. Information on viral tropism in HIV-1 seroconverters is scarce, as is the relationship with transmission of drug-resistant viruses.**

***Methods*: All consecutive HIV-1 seroconverters seen between January 1997 and December 2005 in 17 Spanish hospitals were retrospectively analysed. V3 loop amino acid sequences derived from plasma RNA at the time of initial diagnosis were used to predict co-receptor usage. Major drug resistance mutations, plasma HIV RNA, CD4 counts and HIV subtype were considered for subsequent analyses.**

***Results*: A total of 296 HIV-1 seroconverters were identified (84% male; median age 30 years; 61% homosexual men). Median estimated time from infection was 7 months (interquartile range, 3–11). Primary drug resistance mutations were seen in 12.5%, being 9.5% for nucleoside reverse transcriptase inhibitors (NRTI), 4.4% for non-NRTI (NNRTI) and 3% for protease inhibitors (PI). Twenty-four (8.1%) carried non-B subtypes. HIV tropism could be characterized in 203 seroconverters (69%). X4 viruses (either pure or dual/mixed R5/X4) were recognized in 35 (17.2%). There was no association between HIV tropism and mean plasma HIV RNA (4.5 versus 4.4 log copies/mL in R5 versus X4, respectively; $P = 0.45$) or mean CD4 counts (594 versus 554 cells/mm^3, respectively; $P = 0.48$). The proportion of X4 viruses did not differ in patients infected with wild-type or drug-resistant viruses (17% versus 18%, $P = 1$). Intravenous drug users tended to show X4 viruses more frequently than individuals infected by sexual relationships (35.7% versus 16.5%, respectively; $P = 0.073$). After 12 months of follow-up in 78 seroconverters who did not start antiretroviral therapy, more pronounced increases in plasma HIV RNA (+5056 versus −3430) and declines in CD4 cell counts (−126 versus −60) were seen in X4 compared with R5 carriers.**

***Conclusions*: A significant proportion of recent HIV-1 seroconverters harbour X4 viruses (17.2%), without any evidence of association between co-receptor usage, transmission of drug-resistant viruses and HIV subtype.**

Keywords: HIV tropism, seroconversion, HIV-1, seroconverters

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Introduction

Since the publication of the first report proving that drug-resistant HIV-1 could be efficiently transmitted,[1] surveillance of drug resistance in antiretroviral-naive chronically HIV-infected individuals or recent seroconverters has provided relevant information about the extent of drug resistance in a geographical region and trends over time.[2–8] Moreover, it has allowed us to monitor the spread of new drug-resistant variants within a community [8,9] and track the source of new infections.[10,11]

The overall prevalence of primary HIV-drug resistance in Western countries is currently around 10–15%, with some differences between regions and time periods.[2–8] In Spain, studies conducted over the last decade have shown a steady decline in the rate of genotypic resistance among recent HIV-1 seroconverters between 1997 and 2000.[7] The prevalence has remained fairly stable since then.[11] Surveys among recently HIV-1-infected persons are of particular interest, considering that some drug resistance mutations may become undetectable over time and due to the implications of primary drug resistance for the design of first-line therapies.[9,12,13]

Different classes of entry inhibitors are currently being tested to be part of the antiretroviral armamentarium. Enfuvirtide, a fusion inhibitor, has been the first molecule within this family to obtain approval.[14] Co-receptor antagonists are in the late phases of clinical development, although the development of these compounds (i.e. aplaviroc) has been halted because of safety concerns. CCR5 antagonists inhibit HIV binding to CCR5, preventing the virus from entry into target cells.[15,16] In general, most HIV variants isolated from drug-naive, chronically HIV-infected individuals use CCR5 along with CD4 to gain entry into cells.[17] On the contrary, viruses able to use CXCR4 co-receptors tend to emerge later over the course of HIV infection, being recognized in nearly half of patients in advanced disease stages.[18] Given their mechanism of action, the determination of HIV tropism before the introduction of co-receptor antagonists has been mandatory so far. More epidemiological studies assessing the prevalence of HIV-tropic variants in different populations are needed to identify the most suitable candidates for these new compounds. Studies assessing the role of current antiretroviral drugs and/or resistance mutations on virus co-receptor usage are particularly needed, since these compounds will be often used in antiretroviral-experienced patients and/or in subjects with drug-resistant viruses. Herein, we have assessed the prevalence of virus co-receptor usage in a large cohort of recent HIV seroconverters in Spain and their possible association with drug resistance mutations, HIV subtypes, viral load and CD4 counts.

Patients and methods

Study population

All consecutive newly HIV-1-infected individuals seen between January 1997 and December 2005 in 17 different hospitals distributed across Spain were examined. Subjects with recent HIV seroconversion were defined according to the following criteria: (i) individuals with detectable plasma HIV- RNA together with negative or indeterminate HIV antibody test with or without accompanying typical symptoms; (ii) reactivity using the AXSYM HIV Ag/Ab Combo assay (Abbott Laboratories, Madrid, Spain), with positive HIV p24 antigen detection and negative antibodies confirmed by WB or (iii) seropositivity for HIV-1 infection (reactive ELISA and western blot) being negative on a previous test performed within the prior 12 months.

Sociodemographic data were recorded for each individual using a questionnaire and from hospital clinical charts. Plasma HIV- RNA was measured using the third generation bDNA assay (Versant v3.0, Bayer, Barcelona, Spain), and CD4 counts were determined by flow cytometry (Coulter, Madrid, Spain). For a subset of patients, viral load and CD4 counts were also available 1 year after HIV diagnosis and were used for longitudinal analyses. The study was approved by the Ethics Committees of the participating centres.

Drug resistance mutations

Drug resistance mutations were examined on plasma specimens at the time of initial diagnosis. Genetic sequence analyses of both HIV-1 reverse transcriptase (RT) and protease genes were carried out in plasma using the Viroseq HIV-1 kit (Abbott Laboratories, Madrid, Spain) and an automatic sequencer (ABI Prism 3100; Celera Diagnostics, Madrid, Spain) following manufacturer's instructions. Analyses were conducted including major or primary drug resistance mutations recorded in the latest International AIDS Society-USA panel list (www.iasusa.org, last update in September 2006).[19]

V3 sequence analysis and viral tropism determination

Determination of HIV-1 tropism was retrospectively performed in those individuals with enough plasma stored at −80°C for further genetic characterization on the HIV-1 *env* gene. Genotypic V3 analyses were performed using an RT–PCR, with E80 (5′-CCA ATT CCC ATA CAT TAT TGT G-3′) and E105 (5′-GCT TTT CCT ACT TCC TGC CAC-3′) as outer primers. Subsequently, a nested PCR with ES7 (5′-CTG TTA AAT GGC AGT CTA GC-3′) and E125 (5′-CAA TTT CTG GGT CCC CTC CTG AGG-3′) as inner primers was made. Conditions for PCR reactions were as follows: 48°C for 45 min; 94°C for 2 min; 35 cycles at 94°C for 15 s, 55°C for 30 s and 72°C for 30 s and 72°C for 7 min for the RT–PCR reaction. Then, 94°C for 3 min; 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min and 72°C for 7 min for the nested PCR reaction. PCR amplicons were purified using High Pure PCR Product Purification Kit (Roche, Mannheim, Germany) and directly sequenced in the ABI PRISM 3100 Genetic Analyser using the ABI PRISM Rhodamine Terminator reaction kit (Applied Biosystems, Foster City, CA, USA). Nucleotide sequences were edited with the Sequence Navigator software (Applied Biosystems).

For the prediction of HIV tropism, bioinformatic score methods based on support vector machines (SVM) were used.[16] Basically, this procedure predicts HIV-1 co-receptor usage from the net charge of the V3 loop amino acid sequence of *env*. The tool is freely available at the website: http://genomiac2.ucsd.edu:8080/wetcat/. A positive predictive value of 90% has been reported for SVM methods using phenotypic assays as reference.[20] In a similar population, a phenotypic tropism assay (Phenoscript-ENV Tropism Recombinant assay, Eurofins-Viralliance®, Paris, France) was used to validate the genotypic results, as previously described.[21,22]

Phylogenetic analyses

For subtyping, *pol* sequences from recent seroconverters were aligned with HIV-1 group M reference sequences (http://www.hiv.lanl.gov/content/hivdb/SUBTYPE_REF/Table1.htlm) using the CLUSTAL

X method (MegAlign, Lasergene, DNASTAR Inc., Madison, WI, USA). Phylogenetic analyses were performed using the PHYLIP software package (version 3.5c; J. Felsenstein, University of Washington, Seattle, WA, USA). Evolutionary distances were estimated using Dnadist (Kimura two-parameter method), and phylogenetic relationships were determined using the neighbour-joining method.

Statistical analyses

Baseline characteristics of the study population were recorded as percentages, mean ± SD or median values and 25–75% interquartile ranges (IQR). The rate of drug resistance mutations and the proportion of patients infected by X4 or R5 viruses were recorded as absolute numbers and percentages. The Student's *t*-test was used to compare quantitative variables, whereas the χ^2 test was used to compare categorical parameters. Non-parametric tests were used to assess the significance of any association between R4 tropism and viral load and/or CD4 counts at baseline and after 12 months of follow-up. Differences were considered as significant if *P* values were below 0.05. All reported *P* values were two-sided.

Results

A total of 296 recent HIV seroconverters were identified during the 9 year study period. Their median age was 30 years; 84% were men, most of whom had been infected through homosexual relationships. The median estimated time from exposure to the initial diagnosis of HIV infection was 7 months (IQR, 3–11). Other baseline characteristics of the study population are recorded in Table 1.

The overall rate of primary drug resistance mutations was 12.5% (37/296). By antiretroviral drug class, drug resistance was as follows: 9.5% (28) for NRTI, 4.4% (13) for NNRTI and 3% (9) for PI. The most frequent changes in the RT gene were at position 215, with revertant forms found in 11 and 215Y in five individuals. Other common changes were M41L ($n = 11$), V118I ($n = 6$), M184V ($n = 2$), K103 N ($n = 11$) and Y181C ($n = 2$). At the protease gene, the most common resistance mutations were M46I/L ($n = 4$), V82A ($n = 5$) and L90M ($n = 4$).

Table 1. Main characteristics of the Spanish HIV-1 seroconverter cohort ($n = 296$)

Male gender	248 (83.7%)
Median age (years)	30 (26–36)
Risk group	
homosexual men	180 (60.8%)
heterosexuals	58 (19.6%)
iv drug users	32 (10.8%)
blood transfusion	1 (0.3%)
unknown	25 (8.5%)
Median time from infection (months)	7 (3–11)
Median CD4 count (cells/mm^3)	571 (398–732)
Median CD4 count (%)	26 (19–34)
Median viral load (log HIV RNA copies/mL)	4.67 (4.1–5.1)
Drug resistance mutations	
NRTI	28 (9.5%)
NNRTI	13 (4.4%)
PI	9 (3%)
any	37 (12.5%)
HIV-1 non-B subtypes	24 (8.1%)
X4 viral tropism (pure or dual/mixed R5/X4)[a]	35 (17.2%)

Percentages or IQR are shown in parentheses.
[a]Data available from only 203 patients.

Phylogenetic analyses identified 24 samples with non-B subtypes (8 CRF14_BG, 4 CRF03_AG, 3 CRF12_BF, 3 C, 3 G, 2 F and 1 A). It is noteworthy that all individuals newly infected with non-B variants were identified during the last 3 years. Moreover, acquisition of HIV-1 had occurred following needle sharing in all subjects infected with CRF14_BG, whereas other non-B variants had been acquired through heterosexual intercourses. In contrast, most seroconverters infected with HIV-1 clade B were homosexual men ($P < 0.001$).

HIV-1 tropism could be estimated using V3 genetic sequences generated in 203 patients (69%). Insufficient plasma ($n = 88$) or assay failure ($n = 5$) precluded to drawn results in the remaining 203 patients. In 14 patients, all carrying non-B subtypes, V3 sequences could not be generated or were considered inadequate for viral co-receptor assessment. Large genetic heterogeneity in the viral envelope gene most likely explained it. Overall, the proportion of patients infected with X4 viruses (either pure or dual/mixed X4/R5) was 17.2% (35/203). Primary drug resistance mutations were seen in 10.8% (22/203) of the study population with known co-receptor usage. Overall, 5% (10/203) were infected with non-B subtypes. A total of 35 individuals, from a similar population, with results of HIV tropism inferred from V3 sequences could be tested using a phenotypic recombinant tropism assay, and the results were concordant in 30 (86%) cases. In the remaining five subjects, the phenotypic test showed the presence of X4/R5 dual/mixed viruses, whereas the genotypic analyses concluded that there were only R5 viruses. Thus, overall, the concordance between genotypic and phenotypic results of the tropism tests used in this study was good.

The distribution of X4 viruses did not differ comparing subjects with acute HIV-1 infection (6/34) and the rest of recent seroconverters, including those with more than 6 but less than 12 months from initial exposure (12/67). None of the 10 patients infected with non-B subtypes harboured X4 viruses ($P = 0.14$). There was no association between HIV tropism and mean plasma viral load (being 4.4 versus 4.5 HIV RNA log copies/mL in X4 and R5, respectively; $P = 0.7$). Moreover, mean CD4 counts did not differ significantly between patients harbouring X4 and R5 viruses (554 versus 595 cells/mm^3, respectively; $P = 0.5$). Finally, the prevalence of drug resistance did not differ in patients with X4 and R5 viruses (11.8% versus 11%, respectively; $P = 1$). Neither was an association detected between specific drug resistance mutations and the presence of X4 variants (Table 2). However, individuals who acquired HIV through intravenous (iv) drug use tended to show X4 viruses more frequently than subjects infected through sexual relationships (35.7% versus 16.5%, respectively; $P = 0.072$).

A total of 106 recent seroconverters with known co-receptor usage completed a further 12 months of follow-up and had complete information recorded quarterly on viral load and CD4 counts. Overall, 28 (26.4%) of them fulfilled criteria to initiate antiretroviral therapy, following international guidelines in place at each time point. For the remaining 78 seroconverters, plasma HIV RNA increased a median of 5056 (IQR, −18 310 to

Table 2. Main differences between HIV-1 recent seroconverters infected with X4 or R5 tropic viruses

	R5 ($n = 168$)	X4 ($n = 35$)	*P* value
Median age (years)	31	33	0.172
Male gender (%)	90.4	85.7	0.407
Estimated length of HIV infection (months)	7.8	7.1	0.416
Route of infection (%)			
sexual	84.5	16.5	0.073
iv drug use	64.3	35.7	
Mean viral load (HIV RNA log copies/mL)	4.5	4.4	0.451
Mean CD4 count (cell/mm^3)	595	554	0.489
Patients with HIV-1 non-B subtypes (%)	6.1	0	0.214
Patients with drug-resistant viruses (%)	11	11.8	1

41 492) copies/mL in X4 carriers, whereas it declined a mean of 3430 (IQR, −48464 to 5679) copies/mL in subjects with R5 variants ($P = 0.092$). Consistent with this trend in viral load changes, median CD4 declines at 12 months of follow-up were more pronounced in X4 than in R5 carriers (126 versus 60 cells/mm^3, respectively; $P = 0.696$) (Table 3).

Discussion

This study assessed the prevalence and clinical correlates of CXCR4 tropism in a relatively large population of recent HIV seroconverters. The proportion of X4 viruses and R5/X4 dual tropic viruses in this population was around 18%, a rate quite similar to that reported in studies conducted in drug-naive, chronically HIV-infected individuals.[17,18,23,24] To our knowledge this is the first description of HIV-1 co-receptor usage in a large group of individuals with recent seroconversion. Given that it is generally believed that HIV transmission, at least following vaginal or rectal sexual intercourse, is largely dependent on initial infection of cells harbouring CCR5,[25,26] our results are somewhat unexpected. The pivotal role of R5 viruses as responsible for most initial HIV infections is supported by the fact that individuals homozygotes for the Δ32 CCR5 deletion seem to be 'resistant' to HIV-1 infection,[27,28] with only anecdotal reports of infections occurring by X4 viruses.[29,30]

In our study, recent HIV-1 seroconverters with X4 viruses did not show higher plasma HIV-RNA or lower CD4 counts at the time of initial diagnosis than individuals with R5 viruses. However, the subset of 78 subjects who completed 12 months of follow-up without undergoing antiretroviral therapy showed dichotomous behaviour; X4 carriers showed an increase in plasma viraemia, whereas subjects with R5 viruses showed a decline. Accordingly, more pronounced CD4 declines were seen in the former group compared with the latest. The relatively small size of the study population most likely prevented statistical significance from being reached and a larger group of patients is required to confirm these data. Altogether, these findings are consistent with the postulated increased cytopathic effect of X4 compared with R5 viruses,[31] as well as with its higher replication[32] and accelerated progression to AIDS.[23,33,34]

The prevalence of X4 viruses among individuals who acquired HIV parenterally (all but one iv drug users in our cohort) was more than doubled compared with individuals exposed through sexual relationships (35.7% versus 16.5%, respectively; $P = 0.072$). The limited size of the study population most likely prevented statistical significance from being reached. A similar finding has recently been noticed by others.[17] The mucosal epithelium of the vagina and ectocervix as well as the glans penis and inner foreskin in men consists of stratified squamous epithelial cells interspersed with immature Langerhans cells that express CD4 and CCR5, on their surface, favouring infection by R5 viruses.[35] The expression of CCR5, but not CXCR4, on intestinal epithelial cells may also be relevant to the preferential transmission of R5 viruses via the rectal route.[36] However, during HIV infection via the blood, as in injection drug users sharing needles or haemophiliacs, the size of the inoculum is larger and the target cells different, allowing X4 viruses to establish infection more easily.

Transmission of drug-resistant HIV-1 occurs in 10–15% of newly infected individuals in Western countries.[2–9] Given that virological failure may occur more frequently in subjects treated with regimens including drugs for which resistance is present,[8,12,13] current guidelines recommend drug resistance testing before

Table 3. Evolution of virological and immunological parameters in 106 HIV-1 recent seroconverters during 12 months of follow-up

	R5 ($n = 88$)	X4 ($n = 18$)	*P* value
Antiretroviral treatment (%)	23 (26)	5 (27.7)	0.356
ΔCD4 (cells/mm^3)[a]	−60 (−172/+42.7)	−126 (−181/+23)	0.696
ΔPlasma HIV RNA (copies/mL)[a]	−3430 (−48464/+5679)	+5056 (−18310/+41492)	0.092
ΔPlasma HIV RNA (log copies/mL)[a]	−0.14 (−0.45/+0.11)	+0.1 (−0.37/+0.33)	0.288

[a]Only for those individuals without antiretroviral therapy. Results are expressed as medians and IQR.

initiating antiretroviral therapy. Overall, drug-resistant viruses tend to be less fit than wild-type strains, and some resistance mutations may compromise virus replication more than others, which might explain their differential transmission efficiency.[37,38] However, recent reports have highlighted that some patients failing antiretroviral therapy may show highly replicative X4 viruses despite carrying multiple resistance mutations[32] and that efficient transmission of drug resistance may occur with X4 viruses.[10] In such cases, hypothetically, CXCR4 co-receptor usage could provide an advantage for replication and transmission to drug-resistant strains. If so, an association between X4 tropism and drug resistance might be recognized in recent HIV-1 seroconverters. This was not confirmed in our study, since X4 viruses were equally represented in patients who acquired HIV-1 with or without drug resistance. Neither any association with some specific drug resistance mutations could be noticed. Our data in recent HIV seroconverters are in agreement with those obtained in a large study recently carried out in chronically HIV-infected individuals with and without prior antiretroviral exposure,[18] in which the prevalence of X4 viruses did not differ in patients with and without resistance mutations.

Our study could not answer appropriately whether an association between HIV-1 subtypes and co-receptor usage exists, since the proportion of patients with non-B clades in our cohort was too small. Nevertheless, none of the 10 individuals with non-B viruses harboured X4 variants. Although an association between X4 tropism and specific subtypes (i.e., clade D and CRF14_BG) has been proposed in some studies,[39,40] it has not been confirmed by others.[18] Of note, all five subjects with CRF14_BG and one with clade D in our study carried R5 viruses.

Another potential limitation of our study is that HIV tropism was estimated using a bioinformatic tool which predicts co-receptor usage based on genotypic data rather than by using phenotypic assays. Some rapid, high-throughput recombinant co-receptor phenotype assays have recently been developed, and at least two are now commercially available,[16] the Monogram Biosciences PhenoSense HIV entry assay[41,42] and the Eurofins-Viralliance Tropism Recombinant test.[21,22] Far from perfect, their disagreement is substantial[43] and the proportion of samples for which results cannot be obtained is still significant, particularly when testing non-B subtypes.[18,21,22] Given that HIV-1 tropism is largely driven by the amino acid charges within the third hypervariable (V3) region of gp120, sequence data have been used to infer tropism behaviour with remarkable success.[16,44,45] The vector machine system (VMS) we used in our study is currently among the best in terms of specificity (but less of sensitivity) for X4 viruses.[46] In fact, a subset of 35 individuals tested in parallel with the VMS genotypic software and the Eurofins-Viralliance assay showed highly concordant results (86%). In fact, only the five specimens with disagreement were shown to have X4/R5 dual viruses in the phenotypic test when the genotypic analyses predicted the presence of R5 viruses alone. Sampling variability in the genetic amplification process could somewhat explain this discordance.[47] Taking into account these findings, our estimates on the prevalence of X4 viruses should be considered as conservative. It is noteworthy that the 18% prevalence of X4 in recent seroconverters is very similar to that reported in chronically, drug-naive HIV-1-infected patients.[18,23,24] Therefore, we are confident about the lack of association between co-receptor tropism and drug resistance mutations, CD4 counts and viral load in our population of recent HIV-1 seroconverters.

In summary, a significant proportion of recent HIV-1 seroconverters harbours X4 viruses. This observation may have important clinical and therapeutic implications, since X4 viruses are associated with more rapid disease progression[26] and because CCR5 antagonists might be harmful in this population.[48] Contrary to recent concerns,[10] transmission of drug-resistant viruses does not seem to be associated with HIV-1 co-receptor usage.

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Transparency declarations

None to declare. Q1

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