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Low Frequency of CXCR4-Using Viruses in Patients at the Time of Primary Non-Subtype-B HIV-1 Infection▽

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We used genotypic and phenotypic assays to estimate the frequency of X4/DM viruses in 131 patients infected with non-subtype-B viruses at the time of primary HIV-1 infection (PHI). All patients were enrolled in the French PRIMO Cohort from 1996 to 2007. Most strains belonged to CRF02_AG (51.1%) and subtype A (14.5%). Sixteen viruses (12.2%) were classified as CXCR4 tropic ("X4 strains") by the combined criteria of amino acids 11 and 25 of the V3 loop (11/25) and net charge rules and/or the SVMgeno2pheno$_{10\%}$ algorithm: 6 strains by the combined genotypic rule, 7 by the SVMgeno2pheno$_{10\%}$ algorithm, and 3, clustering in subtype D, by both algorithms. However, only one strain (0.8%), belonging to subtype A, was defined as a dual-tropic (DM) virus by the phenotypic assay. The 67 CRF02_AG strains included 2 classified as X4 strains by the combined genotypic rule (3%) and 2 others classified as X4 strains by SVMgeno2pheno$_{10\%}$ (3%), but none of these 4 strains was an X4 or DM strain according to the phenotypic assay. These results suggest that the cellular virus reservoir was established with X4 strains in very few non-subtype-B-infected patients at the time of PHI. Genotypic predictions can overestimate the proportion of non-subtype-B X4 viruses at PHI.

Human immunodeficiency virus type 1 (HIV-1) can be characterized by the host chemokine coreceptor that it uses to enter CD4-expressing cells. HIV-1 variants usually bind to the CCR5 chemokine coreceptor early in the course of disease. These are "R5" viruses (3, 31, 48). Viruses that use another chemokine coreceptor, CXCR4, are "X4" viruses, and they emerge later in HIV infection. They can account for up to 40 to 50% of all viruses in heavily treated patients with advanced disease (1, 32). The presence of X4 viruses has been associated with accelerated disease progression and a precipitous loss of CD4 T cells (27, 29, 40). A recent Swiss study suggested that the presence of X4 strains and the X4-specific virus load strongly predict clinical disease progression during combined antiretroviral therapy (cART), in addition to the CD4 T-cell count or viral load (44). This potential correlation between virus tropism and disease progression has important clinical implications. The development of coreceptor CCR5 antagonists for treating retroviruses and the lack of a virological response by patients infected with X4 or dual/mixed (X4/DM) viruses have increased the need to determine HIV-1 tropism.

Recent studies have found the frequency of X4/DM dual-tropic strains in plasma samples from recently infected patients in the United States and Spain to be from 3.2% to 17.5% (14, 15, 16). Similarly, we found 15.9% (95% confidence interval [CI], 12.3% to 19.5%) strains of X4/DM viruses in 390 HIV-1 subtype B-infected patients diagnosed at the time of primary HIV-1 infection (PHI) in France from 1996 to 2007 (18).

One of the major challenges of determining tropism is to select the best method for identifying coreceptor usage. HIV coreceptor usage is most commonly determined with a recombinant phenotype assay in clinical studies (28, 45). Bioinformatic tools based on the virus genotype may also be able to predict coreceptor usage. They are faster, less expensive, and more suitable for studies of a large number of patients than are phenotypic recombinant assays. Each available genotypic test is adequately specific but not very sensitive for detecting X4/DM or X4 variants. An overall concordance of 71.2 to 92% between genotypic and phenotypic assays has been reported (8, 15, 37, 41). However, most of these studies included HIV-1 subtype B strains. Genotypic algorithms may not be suitable for predicting the tropism of non-subtype-B HIV-1 strains (20). Two recent studies demonstrated that genotypic tests performed well for predicting the coreceptor usage of CRF02_AG and subtype C strains (36, 38), but no study has examined the correlation between genotypic and phenotypic tests for predicting the tropism of non-subtype-B HIV-1 at the time of PHI. The French PRIMO Cohort contained a large proportion of patients infected with a non-subtype-B virus (25.5% in 2005 to 2006) (6).

We have therefore estimated the frequency of X4/DM viruses in 131 patients infected with non-subtype-B viruses at the time of PHI. All of them were enrolled in the French PRIMO Cohort from 1996 to 2007. We also studied the concordance between genotypic and phenotypic assays for

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predicting the tropism of non-subtype-B viruses in these patients.

MATERIALS AND METHODS

Study population. The patients studied presented with PHI when enrolled in the multicenter ANRS PRIMO Cohort (22). The PRIMO study protocol was approved by the Paris Cochin Ethics Committee, and all subjects gave their written informed consent. Subjects diagnosed during or soon after PHI, whether symptomatic or not, were enrolled. Recent infection was confirmed by (i) a negative or indeterminate HIV enzyme-linked immunosorbent assay (ELISA) result together with a positive antigenemia or plasma HIV RNA result, (ii) a Western blot (WB) profile compatible with ongoing seroconversion (incomplete WB with an absence of antibodies to *pol* proteins), or (iii) an initially negative test for HIV antibodies followed within 6 months by a positive HIV serology. The date of infection was estimated as the date of symptom onset minus 15 days, the date of the incomplete WB minus 1 month, or the midpoint between a negative and a positive ELISA. The interval between the estimated date of infection and enrollment could not exceed 6 months. Each patient underwent a physical examination at enrollment, and blood samples (plasma and peripheral blood mononuclear cells [PBMCs]) were collected for immunological and virological studies at the time of the enrollment in the cohort. No patient had received antiretroviral drugs at the time of inclusion.

V3 *env* sequences. Total DNA was extracted from frozen PBMCs with the QIAamp DNA minikit (Qiagen SA, Courtaboeuf, France). The *env* (340 bp) C2V3 regions were then amplified by using ED3/ED12 as outer primers and Env7/ED33 as inner primers (13). Each PCR mixture contained 1 μg of template DNA, 0.2 μM each primer in Expand high-fidelity buffer (5×) with 75 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate (dNTP), and 2.5 U of Expand high-fidelity *Taq* polymerase (Roche Applied Science, Mannheim, Germany) in a final volume of 50 μl. The amplified products were separated by electrophoresis and purified by using a QIAquick PCR purification kit (Qiagen). Nucleotide sequences were obtained by directly sequencing the amplified DNA using the inner primers of each PCR and Big Dye Terminator V1.1 (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Electrophoresis and data collection were performed with an ABI 3130 genetic analyzer sequencer (Applied Biosystems), and alignments were carried out by using Sequence Navigator software.

Phylogenetic analysis of the V3 region. The HIV-1 subtype was systematically determined after phylogenetic analysis of the V3 sequences. Phylogenetic relationships between V3 *env* sequences were estimated by comparing sequences with previously reported sequences representative of group M, including reference sequences of subtypes, sub-subtypes, and all the circulating recombinant form (CRF) sequences available in the HIV database or GenBank (until CRF43) (http://www.hiv.lanl.gov/content/sequence/HIV/CRFs/CRFs.html). Sequences were aligned by using the Clustal X program (42). Pairwise evolutionary distances were estimated by using Kimura's two-parameter method. Phylogenetic trees were constructed by a neighbor-joining method (neighbor program implemented in the Phylip package). The reliability of the tree topology was estimated from 1,000 bootstrap replicates. TreeView Win16 was used to draw trees for illustration (34).

Genotypic methods for determining virus tropism. We determined the HIV-1 coreceptor usage of each sample by two genotypic methods. One method was the SVMgeno2pheno algorithm (available at http://coreceptor.bioinf.mpi-sb.mpg.de/cgi-bin/coreceptor.pl), used with a false-positive rate of 10%. The other method was a genotypic rule based on amino acid residues at positions 11 and 25 and the overall net charge of V3 (4, 10, 17). One of the following criteria was required for predicting X4 coreceptor usage: (i) R or K at position 11 of V3 and/or K at position 25, (ii) R at position 25 of V3 and a net charge of ≥+5, or (iii) a net charge of ≥+6 (36–38). The V3 net charge was calculated by subtracting the number of negatively charged amino acids (D and E) from the number of positively charged ones (K and R).

Phenotypic method for determining virus tropism. A region spanning gp120 and the ectodomain of the gp41 *env* gene of plasma HIV-1 RNA was amplified by reverse transcription-PCR. The phenotype of HIV-1 coreceptor usage was determined by using a recombinant virus entry assay, as previously described (35). The sensitivity of the assay for detecting minor amounts of CXCR4-using viruses was 0.5% (35).

Nucleotide sequence accession numbers. Virus sequences were submitted to GenBank with the following accession numbers AM851091, GQ409975 to GQ409981, GQ409985, GQ409988 to GQ409991, GQ409993 to GQ409994, GQ409997, GQ409999 to GQ410007, GQ410009 to GQ410010, GQ410013, GQ410015 to GQ410017, GQ410019, and GU988902 to GU989000.

TABLE 1. Baseline characteristics of the 131 patients infected with non-subtype-B HIV-1

Parameter[a]	Value
No. (%) of male patients	91 (69.5)
No. (%) of patients in risk group	
MSM	55 (41.9)
Heterosexual	69 (52.7)
i.v. drug user	1 (0.8)
Other	6 (4.3)
Median time since infection (days) (range)	35.5 (18–169)
Median CD4 cell count (cells/mm^3) (range)	523 (128–1,393)
Median HIV RNA (log_{10} copies/ml) (range)	4.84 (2.65–8.33)
Median HIV DNA (log_{10} copies/10^6 PBMCs) (range)	3.20 (1.84–4.55)[b]

[a] i.v., intravenous.
[b] Data not available for five patients.

RESULTS

A total of 870 patients were enrolled in the French ANRS PRIMO Cohort between 1996 and December 2007. Of these patients, 207 were infected with a non-subtype-B strain based on the phylogenetic analysis of the *env* gene. This substudy was carried out with 131 patients (Table 1) because samples were not available for the other 76 patients. The median time between infection and enrollment was 35.5 days (range, 18 to 169 days). Six strains (4.6%) were resistant to at least one class of antiretrovirals, and two viruses were resistant to two classes (1.5%). The distribution of the viruses into subtypes/CRFs was as follows: 19 subtype A (14.5%), 4 subtype C (3.1%), 6 subtype D (4.6%), 8 subtype F (6.1%), 9 subtype G (6.9%), 2 CRF01_AE (1.5%), 67 CRF02_AG (51.1%), 2 CRF09_cpx (1.5%), and 2 CRF27_cpx (1.5%) strains. Twelve strains (9.2%) remained undetermined, as they did not cluster with any known subtype or CRF. We have previously sequenced the full lengths of four of them: three subtype B/C/U recombinant viruses and one subtype A/K/U recombinant virus (19).

We found 16 viruses (12.2%) classified as X4/DM by at least one of the two genotypic rules/algorithms. Of these, 10 strains (7.6%) were classified as X4/DM with the $SVMgeno2pheno_{10\%}$ algorithm: they belonged to subtype A ($n = 1$), subtype D ($n = 3$), CRF01_AE ($n = 2$), CRF02_AG ($n = 2$), and CRF27_cpx ($n = 1$); the last one remained undetermined. We identified 9 strains (6.9%) as being X4/DM strains by the combined genotypic rule: 1 subtype A strain, 4 subtype D strains, 2 CRF02_AG strains, 1 subtype G strain, and 1 undetermined strain. Three strains (2.3%) were classified as X4/DM with both $SVMgeno2pheno_{10\%}$ and the combined genotypic rule: all of them belonged to subtype D.

Only one strain (0.8%), which clustered into subtype A, was defined as a dual-tropic virus by the phenotypic assay: it was classified as a X4/DM strain by the combined genotypic rule but as an R5 strain by $SVMgeno2pheno_{10\%}$. This strain was isolated from a patient infected after heterosexual contact.

The agreement between the genotypic classification based on the combined criteria of the 11/25 and net charge rules and the $SVMgeno2pheno_{10\%}$ tool and the phenotype according to the HIV subtype are summarized in Table 2. Two of the 67 CRF02_AG strains were classified as X4 tropic by the combined genotypic rule (3%), and two others were classified as

TABLE 2. Genotypic prediction of HIV-1 tropism and observed phenotypes for predicting CXCR4 usage of CRF02_AG, subtype A, subtype D, and other non-subtype-B strains

Prediction method	No. of strains of indicated predicted phenotype							
	CRF02_AG (n = 67)		Subtype A (n = 19)		Subtype D (n = 6)		Other subtype (n = 39)	
	R5	R5X4/X4	R5	R5X4/X4	R5	R5X4/X4	R5	R5X4/X4
Combined 11/25 and net charge rules								
R5	65	0	18	0	2	0	37	0
X4	2	0	0	1	4	0	2	0
Geno2pheno$_{10\%}$[a]								
R5	65	0	17	1	3	0	35	0
X4	2	0	1	0	3	0	4	0

[a] Value of the fixed false-positive rate.

X4 tropic by SVMgeno2pheno$_{10\%}$ (3%), but none of these four strains was identified as an X4 strain by the phenotype assay.

DISCUSSION

The phenotypic assay identified very few (0.8%) X4/DM viruses in the 131 patients infected with a non-subtype-B strain and diagnosed at the time of PHI in France from 1996 to 2007. This is one of the largest series of such patients examined to date. The genotypic rules identified 12.2% of strains (16/131) as being X4/DM viruses: 7 strains were classified as X4/DM strains by the SVMgeno2pheno$_{10\%}$ algorithm, 6 were classified as X4/DM strains by the combined genotypic rule, and 3 were classified as X4/DM strains by both algorithms. Because only one strain was dual tropic with the phenotypic assay, we could not evaluate the sensitivity of the genotypic methods to predict viral tropism. Further studies are needed to perform this evaluation.

Previous studies have shown that the combined genotypic algorithm is better than the 11/25 and net charge rules used separately to predict HIV-1 coreceptor usage in subtype B viruses (11, 37). A recent report found that genotypic tools were not very sensitive for detecting X4 variants in non-B subtypes. However, no details of their performance for a particular subtype were given (20). In another study, Raymond et al. found that the SVMgeno2pheno$_{10\%}$ tool lacked sensitivity for predicting the CXCR4 usage of CRF02_AG strains, whereas the combined 11/25 and net charge rule criteria were equally good at predicting the CXCR4 usage of both CRF02_AG and subtype B strains (36, 37). We could not evaluate the sensitivity of these genotypic tools because none of our patients was infected with an X4-tropic CRF02_AG virus. However, we confirmed their previously reported high specificity for this subtype (97%; 95% CI, 89.6% to 99.6%).

The combined 11/25 and net charge rule criteria seemed to be more sensitive for detecting CXCR4 usage in the 19 subtype A strains than was the SVMgeno2pheno$_{10\%}$ algorithm, but their specificities were similar (≥94%).

As the phenotypic test is complex to implement in a clinical setting, our results suggest that using the combined 11/25 and net charge rule criteria or using the SVMgeno2pheno$_{10\%}$ algorithm can provide reliable information on HIV-1 tropism when testing CRF02_AG or subtype A strains. Genotypic assays can be useful for predicting the tropism of CRF02_AG recombinant subtype and subtype A strains in clinical practice, as these are the predominant non-B subtypes of HIV-1 in Western Europe.

We tried to evaluate the performance of the genotypic algorithms for subtype D viruses, although these have been rarely found in the French PRIMO Cohort, as this subtype is said to be mainly X4 tropic. The algorithms gave different results, but the SVMgeno2pheno$_{10\%}$ tool was more specific. Further studies on more subtype D strains are needed to estimate the validity of such genotypic rules for predicting coreceptor usage for this subtype.

Although some studies found no difference in coreceptor tropism among the different virus subtypes (47), recent reports suggested that syncytium-inducing (SI) or CXCR4-using strains predominate in some subtypes (subtype D and CRF01_AE) (9, 23, 26, 43, 46), while non-SI/CCR5-using strains tend to predominate in some other subtypes (subtypes A and C) (2, 26, 33, 43, 46). The fact that we find no differences between subtypes/CRFs could be due to the small number of strains of each type (except CRF02_AG). However, 20 to 45% of the strains that were classified as X4-tropic viruses by the successive genotypic algorithms belonged to subtype D, and the two CRF01_AE viruses were defined as X4 tropic with the SVMgeno2pheno$_{10\%}$ algorithm. This could be due to the poor sensitivity of the phenotypic assay for detecting minor CXCR4-using virus variants. However, our phenotypic assay detects minor CXCR4-using variants when they account for as little as 0.5% of the virus population, which is substantially better than the analytical sensitivity of direct sequencing (≥10 to 20%). In addition, our study was performed at the time of PHI, when the dominant homogenous population is usually expanding (12, 21). These discordant results could also be due to the poor specificity of the genotypic algorithms for non-subtype-B strains that include few X4 viruses.

We find a smaller proportion of X4/DM strains in patients with an acute infection than the 3.2 to 17.2% reported in previous studies (14, 15, 16, 18). However, those earlier studies included very few non-subtype-B strains: there were no non-subtype-B viruses in the French and American reports and 4 (6.5%) and 10 (5%) viruses in the Spanish studies, which found no X4/DM non-subtype-B viruses. This rarity of X4/DM viruses is in line with what was reported previously for large

cohorts of antiretroviral-naïve chronically infected individuals, in which the proportion of non-subtype-B-infected patients was very low, if it was reported (5, 25, 32).

We performed our study on cell-associated viruses using HIV DNA extracted from PBMCs, which is representative of the virus in the cellular reservoir. In contrast, most studies have carried out tropism tests on HIV RNA extracted from plasma. A recent report by Raymond et al. described the good agreement between HIV-1 tropisms in PBMCs and plasma at the stage of PHI (39). Our results suggest that the cellular virus reservoir is established with X4/DM strains in a very small proportion of patients infected with non-subtype-B viruses at the time of PHI. Further studies are needed to evaluate the virus tropism in the tissues of patients during primary HIV-1 infection, especially in cases where a patient is infected with a X4/DM strain, as Mefford et al. recently reported that bioinformatics prediction programs underestimate the frequency of CXCR4 usage by R5X4 strains in the brain and other tissues (30).

X4/DM strains were found in fewer PRIMO Cohort patients infected with non-subtype B HIV than in patients infected with subtype B, where 14.3% of X4/DM-tropic viruses were identified in PBMC samples using the SVMgeno2pheno$_{10\%}$ algorithm (18). These results could be explained by differences in the demographics and behaviors of the two groups of PRIMO Cohort patients. The patients infected with subtype B strains were mainly native French men who had had sex with men (MSM). Clusters could be frequently isolated from these men in phylogenetic studies. Many of the non-subtype-B-infected patients were heterosexual men and women originally from sub-Saharan countries. We postulate that the subtype B patients had been infected by chronically infected, intensively treated, patients whose strains were more frequently X4/DM tropic.

The difference between the two populations is also supported by the fact that more subtype B group patients were infected with a resistant strain than were non-subtype-B patients during the first decade of the PRIMO Cohort (data not shown). This study, which includes the majority of non-subtype-B-infected patients enrolled since 1996, finds that fewer non-subtype-B-infected patients were infected with resistant viruses (4.6%) than the subtype B-infected patients (15.9%) tested in our previous study (18), which included recently infected patients from the PRIMO Cohort.

There could be another explanation for the differences in the coreceptor usages of the subtype B and non-subtype-B strains isolated from the PRIMO Cohort patients. Several studies have shown that while the V3 region is a major determinant of coreceptor tropism, other regions outside the coreceptor binding site and outside the gp120 surface subunit (in the gp41 transmembrane subunit) can contribute to coreceptor usage (7, 23, 24). These regions, which have yet to be fully characterized, differ between HIV-1 subtypes and influence the susceptibility of certain virus subtypes to use predominantly a CCR5 or CXCR4 coreceptor.

Finally, the lower prevalence of X4-tropic non-subtype-B strains in this report than in our previous study on subtype B-infected patients from the PRIMO Cohort could be due to the smaller number of subjects studied. This could have underestimated the frequency of CXCR4 use in non-subtype-B viruses.

In conclusion, the frequency (0.8%) of X4/DM viruses in patients infected with non-subtype-B strains of HIV-1 is very low at the time of PHI. The use of genotypic methods at this time could overestimate the proportion of non-subtype-B X4-tropic strains.

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