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MAJOR ARTICLE

Presence of CXCR4-Using HIV-1 in Patients With Recently Diagnosed Infection: Correlates and Evidence for Transmission

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Background. The prevalence and correlates of CXCR4-use in recently diagnosed patients and the impact of X4/DM transmission remain largely unknown.

Method. Genotypic coreceptor use determination on the baseline sample of 539 recently diagnosed individuals. Correlation of coreceptor use with clinical, viral and epidemiological data and with information on transmission events as obtained through phylogenetic analysis of protease and reverse transcriptase sequences.

Results. CXCR4-use was predicted in 12 to 19% of the patients, depending on the interpretative cutoff used. CXCR4-use was correlated with lower $CD4^+$ T cell counts and subtype 01_AE infection. No association with viral load was observed. Seven (11%) of 63 transmission clusters and 4 (31%) of 13 donor-source pairs resulted from X4/DM transmission.

Conclusion. The results confirmed the relation between CXCR4-use at diagnosis and low baseline CD4+ T cell counts. Significantly more CXCR4-use was predicted in 01_AE infections, which may impose constraints on the use of CCR5 antagonists in certain regions of the world. Observations from the transmission cluster analysis contradict the hypothesis that R5 viruses are selected at transmission, and support the idea that R5 or X4/DM infections result from a stochastic process.

To enter cells, human immunodeficiency virus type 1 (HIV-1) needs to interact with the CD4 receptor and either of 2 coreceptors: CCR5 or CXCR4. Viruses have the ability to use CCR5 (R5), CXCR4 (X4), or both (dual R5X4). In clinical samples, R5 and X4 viruses are often present together (mixed R5X4). Evolution of the HIV-1 *envelope* (*env*) gene can lead to expansion of coreceptor use from CCR5 to CXCR4 with disease progression [1], but the underlying mechanism remains poorly understood.

Because of the proven association between presence of X4 or dual/mixed (DM) viruses and faster disease progression [2, 3], determination of HIV-1 coreceptor tropism can be used as a prognostic tool. Interest in tropism has currently increased by the availability of the coreceptor blocker maraviroc, which has exclusive activity against R5 viruses. Successful application of this drug requires pretreatment screening to exclude X4/DM presence.

Recent developments in technology for coreceptor tropism determination mainly focus on genotypic assays that rely on the established relationship between the amino acid sequence of part of the HIV envelope called the V3 *loop* and specificity for one of the coreceptors. Combination of population V3 sequencing with bioinformatic tools to predict coreceptor tropism, such as Geno2pheno, is now generally accepted in Europe as a way to define tropism and maraviroc susceptibility in clinical practice [4].

R5 viruses predominate in recent infections [5]. This observation, together with the finding that a deleterious homozygous mutation (Δ32) in the gene coding for CCR5 largely protects against HIV-1 infection [6], resulted in the assumption of a transmission bottleneck that favors R5 strains. Although many potential mechanisms to explain this bottleneck have been suggested

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[7], arguments supporting selective transmission were mainly obtained in vitro. Furthermore, there are indications that selective forces at transmission are at least imperfect. Reports of HIV infection in homozygous Δ32 patients prove that the CCR5 coreceptor is not an absolute requirement for transmission [8, 9], vertical transmission of CXCR4-using viruses has been demonstrated [10, 11], and CXCR4 use has been described in acutely infected individuals [12–16]. However, reports of proven transmissions of CXCR4-using viruses remain extremely rare.

The present study aimed at defining the epidemiology of coreceptor use predicted by genotypic tropism testing in a cohort of patients with newly diagnosed HIV-1 infection consulting the AIDS Reference Center (ARC) in Ghent between January 2001 and December 2009. The first objective was to define correlates for CXCR4 use. The second objective was to assess the potential of X4/DM virus transmission through phylogenetic transmission cluster analysis.

METHODS

Study Subjects

A total of 593 patients were retrospectively selected from individuals consulting the ARC of the University Hospital Ghent, Belgium, between January 2001 and December 2009. Patients signed an informed consent form, had their first consultation after diagnosis in the ARC of Ghent, and had a plasma sample collected within 1 year of diagnosis available. Information on HIV transmission route, sex, age, origin, $CD4^+$ T-cell count, viral load, and the baseline protease (PR) and reverse transcriptase (RT) sequences were retrieved anonymously from the patients' files. Patients were epidemiologically linked when information about the possible source of infection was available. Patients were considered acutely infected when a negative HIV screening result within 1 year of the first positive result was available and chronically infected when the infection could be dated >1 year before presentation. For the majority of the patients, however, information on the presumed infection date was missing. The list of transmitted drug-resistance mutations (DRMs) established by the World Health Organization was used to identify drug resistance [17]. The project was approved by the Ethics Committee of the institution.

CCR5 Genotyping

To determine the presence of the deleterious 32–base pair (bp) deletion in CCR5, a fragment flanking the deletion was amplified from genomic DNA extracted with a QIAamp DNA Blood Minikit (Qiagen). Primers and amplification conditions were depicted from de Roda Husman et al [18] and the reverse primer was fluorescent labeled with FAM to allow analysis of the amplified products on an ABI-Prism 3130XL Genetic Analyzer (Applied Biosystems). Electropherograms were interpreted visually and with the software program Basehopper (http://www.basehopper.be/) to define the amplicon length, as follows: wild type (wt)/wt: 239 bp, wt/Δ32: 239 bp + 207bp, Δ32/Δ32: 207 bp.

Phylogenetic Analysis of the *pol* Gene Sequences

Baseline HIV-1 PR and RT sequences were available for 576 (97%) of the 593 individuals selected. Transmission clusters were identified as described elsewhere [19]. In short, maximum likelihood estimated distances according to the chosen model were used to reconstruct neighbor joining phylogenetic trees in PAUP* v4.0b10 [20]. Bootstrap analysis was performed on 1000 replicates, and clusters with a bootstrap value ≥90 were selected. A more robust method, Bayesian inference implemented with MrBayes software version 3.2 [21], was then used to verify the clusters. Only clusters with a Bayesian posterior probability of 1 were considered to have resulted from transmission events and were selected for further analysis. The average genetic distances for the *pol* and V3 regions were used as surrogate markers for the time passed between the transmission event and sampling.

HIV-1 Subtyping

HIV-1 subtyping was performed using PR and RT sequences and the Smartgene (IDNS) or REGA subtyping tool [22]. Subtype B infection was seen in 344 patients (60%). Within the non-B subtypes, CRF02_AG (11%), CRF01_AE (7%), C (7%), and A (6%) represented >5% of the patients.

Coreceptor Tropism Determination

Genotypic coreceptor tropism determinations were performed on viral RNA extracted from the earliest available EDTA plasma using the High Pure viral RNA kit (Roche Applied Science). V3 population sequencing was attempted for 564 of the 576 patients, as described elsewhere [23], and was successful for 539 (96%). Sequencing products were analyzed on the ABI 3130XL Genetic Analyser (Applied Biosystems). Proofreading was performed with Smartgene (IDNS).

V3 nucleotide sequences were subsequently presented to Geno2pheno for coreceptor tropism prediction [24]. For classification as R5 or X4/DM, 2 false-positive rate (FPR) cutoffs were chosen, 5.75% and 10%, based on reports describing the use of this method to predict maraviroc susceptibility [25], comparison with phenotypic assays [26], and European guidelines for tropism testing [4]. Samples with an FPR above the cutoff are labeled as R5, and samples with an FPR below the cutoff are referred to as X4/DM or CXCR4 using (V3 sequences GenBank accession numbers: JN 407559 to JN 408063).

Phenotypic tropism determination was performed for a selection of patients using the method described recently [27]. *Env*-recombinant viruses were generated by cotransfecting the pNL4-3Δ*env*.Luc plasmid and *env* amplicons from patient virus isolates into HEK293T cells (ATCC). The *Env*-recombinant

Table 1. Characteristics of Patients Classified as Harboring X4/DM or R5 Virus Using 10% or 5.75% FPR Cutoff

	FPR ≤ 10			FPR ≤ 5,75		
	X4/DM	R5	*P*	X4/DM	R5	*P*
Patient Characteristics (n=539)	103	436		64	475	
Age, median (IQR), y (n=539)	38 (31–43)	37 (31–44)	.67	38 (32–42)	37 (31–44)	.94
Race or ethnicity, No. (%) (n=533)						
White	75 (73%)	305 (71%)	.7	47 (73%)	333 (71%)	.7
Black	20 (19%)	106 (25%)	.26	10 (16%)	116 (25%)	.11
Other	8 (8%)	19 (4%)	.16	7 (11%)	20 (4%)	.03
Gender, No. (%) (n=539)						
Male	78 (76%)	320 (73%)	.63	49 (77%)	349 (73%)	.6
Female	25 (24%)	116 (27%)		15 (23%)	126 (27%)	
CCR5 genotype, No. (%)(n=473)						
wt/wt	81 (89%)	333 (87%)	.63	52 (90%)	362 (87%)	.6
wt/Δ32	10 (11%)	49 (13%)		6 (10%)	53 (13%)	
Δ32/Δ32	0 (0%)	0 (0%)		0 (0%)	0 (0%)	
CCR5 genotype white patients only, No. (%) (n=344)						
wt/wt	59 (87%)	230 (84%)	.57	39 (89%)	250 (84%)	.42
wt/Δ32	9 (13%)	44 (16%)		5 (11%)	48 (16%)	
Δ32/Δ32	0 (0%)	0 (0%)		0 (0%)	0 (0%)	
Baseline $CD4^+$ T cell count, Median (IQR), cells/mm^3 (n=537)	360 (160–521) (n=102)	385 (256–581) (n=435)	.012	347 (69–519) (n=64)	386 (255–578) (n=473)	.01
Baseline $CD4^+$ T cell count <200, Median (IQR), cells/mm^3(n=108)	44 (10–161) (n=34)	120 (65–161) (n=74)	.042	29 (3–167) (n=23)	125 (62–161) (n=85)	.009
Viral characteristics						
Baseline Viral Load, Median (IQR), log copies/ml (n=536)	4,6 (4–5)	4,5 (3,9–5)	.27	4,6 (3,8–5,2)	4,5 (3,9–5)	.29
Transmitted Drug Resistance, No. (%) (n=539)						
Yes	13 (13%)	25 (6%)	.01	8 (12%)	30 (6%)	.11
No	90 (87%)	411 (94%)		56 (88%)	445 (94%)	
Infection status, No. (%) (n=144)						
Acute	14 (56%)	75 (63%)	.51	8 (47%)	81 (64%)	.18
Chronic	11 (44%)	44 (37%)		9 (53%)	46 (36%)	
Virus subtype, No. (%) (n=539)						
B (n=323, 60%)	57 (55%)	266 (61%)	.29	37 (58%)	286 (60%)	.71
Non B(n=216, 40%)	46 (45%)	170 (39%)		27 (42%)	189 (40%)	
C	5 (5%)	26 (6%)	.66	3 (5%)	28 (6%)	.69
A	7 (7%)	27 (6%)	.83	2 (3%)	32 (7%)	.41
01_AE	17 (17%)	26 (6%)	<.001	14 (22%)	29 (6%)	<.001
02_AG	8 (8%)	50 (11%)	.3	4 (6%)	54 (11%)	.22
other	9 (9%)	41 (9%)	.83	4 (6%)	46 (10%)	.4
Transmission route, No. (%) (n=442)						
Homosexual contact	54 (62%)	211 (59%)	.65	31 (62%)	234 (60%)	.75
Heterosexual contact	29 (33%)	135 (38%)	.42	16 (32%)	148 (38%)	.43
IVDU	4 (5%)	4 (1%)	.05	3 (6%)	5 (1%)	.05
Other	0 (0%)	5 (1%)	.59	0 (0%)	5 (1%)	>.99

For each parameter, only samples for which the information was available were included in the analysis (eg, information on infection status was known for only 144 individuals [25 X4/DM and 119 R5]).

Abbreviations: DM, dual/mixed; FPR, false-positive rate; IQR, interquartile range; IVDU, intravenous drug use; wt, wild type.

viruses were then used to infect U87.CD4.CCR5 and U87.CD4.CXCR4 indicator cells (AIDS Research and Reference Reagent Program, National Institutes of Health) [28] and luminescence was monitored 48 hours later to quantify infection.

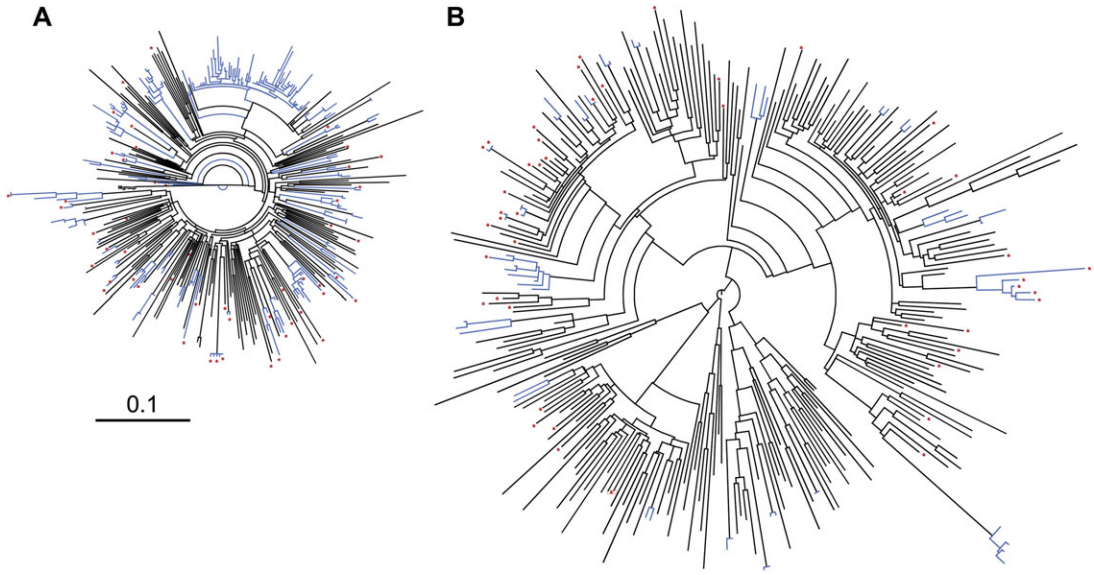

Figure 1. Phylogenetic trees of individuals with subtype B (*A*) and non-B (*B*) infection. Transmission clusters are depicted in blue. Red asterisks indicate the presence of CXCR4-using virus.

Statistical Analyses

Groups were compared using a χ^2 test for categorical variables and the Mann–Whitney *U* nonparametric test for continuous variables. The level of significance was set at $P \leq .05$. All data were analyzed using SPSS 18.0 software (SPSS).

RESULTS

Frequency and Correlates of Predicted CXCR4 Use

Of the 539 patients, 103 (19%) were predicted as CXCR4 using with the 10% FPR cutoff and 64 (12%) with the 5.75% cutoff. The characteristics of the patients according to predicted coreceptor use are shown in Table 1. Whatever the cutoff, no significant differences were seen between the R5 and X4/DM population with regard to age, origin, sex, or CCR5 genotype. Because the CCR5 deletion is rare in nonwhites, the statistical analysis for association between CXCR4 use and CCR5 genotype was also performed after stratification for ethnic origin, but lack of association remained. The only host-specific parameter that differentiated the R5 and X4/DM populations was the baseline $CD4^+$ T-cell count, and this was reflected by lower median CD4 count and higher number of patients with CD4 count <200 cells/mm^3 in the X4/DM population ($P = .01$ and $P < .001$, respectively). No association between coreceptor use and viral load or infection stage was found. A significantly higher number of transmitted drug resistant mutations was seen in the X4/DM viruses with a FPR $\leq$10%, but the association was lost when applying the 5.75% cutoff. X4/DM presence was not associated with the route of transmission, with the exception of intravenous drug use (IVDU), which was associated with higher X4/DM prevalence ($P = .05$). X4/DM prevalence was comparable in subtype B and non-B infections, but analysis of the 4 most represented subtypes revealed a significantly higher number of X4/DM predictions in CRF 01_AE compared with C, A, and CFR02_AG ($P < .001$).

Predicted CXCR4 Use in Transmission Clusters

Phylogenetic analysis of the PR+RT sequences of the 576 patients identified 63 patient clusters with a bootstrap value of $\geq$90 and a posterior probability of 1, considered to represent onward virus transmission (Figure 1). Forty-two clusters, comprising 203 patients, had subtype B infections and 21 (58 individuals) had non-B infections. Virus predicted as X4/DM according to the FPR cutoff of 10% was present on separate branches (55/278; 19.9%) as well as on clustered branches (48/261; 18.4%).

The characteristics of the different types of transmission clusters with regard to tropism prediction are summarized in Table 2. Table 3 lists the information for individual clusters. Using a 10% FPR cutoff, 7 clusters (20 individuals) were predicted to be X4/DM. Two of these were relatively large (5 individuals each). In 38 clusters (167 individuals) only R5 viruses were detected, and in 18 (74 individuals) R5 as well as X4/DM viruses were found (mixed clusters). Subtype B and non-B infections were represented in R5, X4/DM, and mixed clusters. IVDU transmission was reported in mixed and X4/DM clusters, but not in R5 clusters. Infections resulting from homosexual or heterosexual contacts were similarly distributed within X4/DM, mixed, and R5 clusters.

Because of the stringent criteria used to define the phylogenetic clusters, they most certainly represent onward HIV transmission. Surrogate markers such as the genetic distance of the *pol* and

Table 2. Characteristics of Phylogenetic Clusters Classified According to Results of Genotypic Tropism Prediction

	Type of cluster		
	R5	R5 + X4/DM	X4/DM
Clusters, No.	38	18	7
Viral Tropism, No. of patients (%)			
R5 virus	158 (95%)	44 (59%)	0
X4/DM virus	0	28 (38%)	20 (100%)
Unknown	9 (5%)	2 (3%)	0
Cluster size, range, No. of patients	2–63	2–11	2–5
Transmission mode, No. of patients (%)			
Homosexual	19 (50%)	13 (72%)	4 (57%)
Heterosexual	17 (45%)	3 (17%)	2 (29%)
IVDU	0	2 (11%)	1 (14%)
Unknown	2 (5%)	0	0
Viral Subtype, No. of patients (%)			
B	24 (63%)	14 (78%)	4 (57%)
Non B	11 (29%)	4 (22%)	3 (43%)
Unknown	3 (8%)	0	0
Genetic distance, mean ± SD, substitutions/site, %			
PR+RT	0.9 ± 1.3	1.2 ± 1.2	0.7 ± 0.8
V3	2.7 ± 2.8	4.3 ± 4.2	1.2 ± 1.5

Abbreviations: IVDU, intravenous drug use; PR, protease; RT, reverse transcriptase; SD, standard deviation.

V3 sequences were additionally used to estimate the average time of evolution between infection and sampling. In the 7 X4/DM clusters, the average genetic distance between the V3 sequences was 1.2% ± 1.5% (Table 2). Identical V3 sequences were seen in clusters 42, 30, and 48 (Table 3). The average genetic distance for the V3 sequences was higher for the mixed clusters compared with the X4/DM clusters (4.8% ± 4.2 vs. 1.2% ± 1.5; $P = .047$) and the R5 clusters (4.8% ± 4.2 vs. 2.7% ± 2.8; $P = .167$). The genetic distance for the *pol* sequences was 1.2% ± 0.8 in the mixed clusters versus 0.7% ± 1.2 in the X4/DM ($P = .025$) and 0.9% ± 1.2 in the R5 clusters ($P = .111$). The topologies of the mixed clusters did not display a uniform pattern (eg, separate subclustering of R5 or X4/DM, R5 outlier branches in X4 clusters or the reverse). Examples of observed tree topologies are shown in Figure 2.

In-depth investigation of the 7 X4/DM clusters showed that for cluster 42, all 5 members were males infected with a subtype B virus through homosexual contact and diagnosed within 1 year of each other. They had highly homogeneous PR + RT sequences (genetic distance, ≤0.002) and identical V3 sequences. Three of the 5 presented during acute infection and were epidemiologically linked. Viruses isolated from these 5 individuals were reported as DM in the phenotypic assay. The second X4/DM cluster of 5 individuals (cluster 5B) was a cluster of CRF02_AG infections. An epidemiologic link between the members of this cluster was not apparent, but all were males infected through homosexual contact. The average genetic distance for PR + RT and V3 sequences was 3.3% and 2.4%, respectively, indicating longer evolution between the transmission event and sampling. In accordance, the time span between diagnosis for the first and the last members was relatively large (7 years). The viruses isolated from the members of this cluster were scored R5 in the phenotypic assay. The remaining 5 X4/DM clusters (clusters 27, 30, 48, 14B, and 15B, Table 3) each contained 2 individuals. Phenotypic analysis confirmed the presence of X4 or D/M virus in both members for clusters 27, 48 and 14B. For cluster 30, the phenotypic assay scored the viruses as R5, and for cluster 15B, 1 patient was scored R5 and 1 patient was scored X4 (see details below).

CXCR4 Use in Donor-Recipient Pairs

To specifically address the possible role of X4/DM viruses in transmission, clustered pairs of individuals for which sufficient data permitted them to be identified as source and receptor (eg, sexual partners or needle-sharing partners) were selected. Thirteen of the 38 partners retrieved as such showed highly related PR + RT sequences (genetic distance, ≤0.001) and were therefore presumed to represent very recent transmission events (Table 4). In 4 of 13 (31%) an X4/DM virus was predicted in both individuals; in 9 (69%) both individuals were infected with an R5 virus. Two of the 4 X4/DM transmissions resulted from homosexual contacts and 2 from heterosexual contacts. Of the 9 R5 transmissions, 7 were acquired homosexually and 2 heterosexually. For 3 X4/DM transmission pairs (pairs 10, 11, and 12; Table 4), infection with an X4/DM virus was confirmed by phenotypic analysis for both individuals. One of the members of pairs 10 and 11 presented with acute infection.

Transmission pair 13 (cluster 15B, see above) was a heterosexual couple with strong indications for female-to-male transmission, but phenotypic analysis showed the presence of an X4 virus in the male (13A) and an R5 virus in the female partner (13B). The female partner presented with lower baseline CD4 counts than the male partner (60 vs 543 cells/mm^3). Although the V3 sequences obtained from both partners were predicted as CXCR4 using by genotypic analysis, their amino acid sequences differed greatly, and the FPR obtained was lower for the male than for the female partner (0.1% vs 8.7%). Limiting dilution sequencing of viruses isolated from the female partner revealed the presence of 3 distinct V3 sequences (Table 4), of which one (clone 3) was highly similar to the V3 sequence in the male partner and had a much lower FPR. The phenotypic analysis confirmed the viruses from the male partner as X4 but failed to detect CXCR4 use in the sample from the female partner.

Table 3. Detailed Overview of the R5, Mixed, and X4 Transmission Clusters

Cluster ID	Patients, No.	Viral Subtype	Main transmission mode	Interval between first and last diagnosis, y	Genetic distance, mean, substitutions/site, %		GTD per FPR category, No. of patients				PTD, No. of patients		
					PR+RT	V3	≤5.75%	>5.75% – ≤10%	>10%	NA	R5	X4/DM	NA
R5 Clusters (n=38)													
3	10	B	HO	6	1.1	1.8			10				10
19	7	B	HO	5	0.8	0.7			6	1			7
7	5	B	HO	8	0.8	1.5			5				5
15	5	B	HO	4	0.6	4.7			5				5
4	4	B	HO	2	0.7	1.7			4				4
6B	4	06_cpx	HE	5	0.7	3.9			3	1			4
11B	4	UD	HE	5	1.7	2.2			4				4
2	3	B	HO	2	1.0	3.0			3				3
22	3	B	HO	5	0.9	3.1			3				3
25	3	B	HO	<1	0.9	0.9			3				3
24B	3	02_AG	HE	4	7.5	6.0			3				3
39	63	B	HO	8	1.2	5.6			57	6	4		59
19B	3	UD	HE	3	3.2	10.8			2	1			3
5	2	B	HO	<1	<0.1	11.2			2				2
6	2	B	HO/HE	1	0.7	1.0			2				2
11	2	B	HO	<1	0.8	3.0			2				2
12	2	B	HE	<1	0.4	<0.1			2				2
13	2	B	HO	2	1.1	0.3			2				2
17	2	B	HO	<1	0.1	<0.1			2				2
26	2	B	HE	<1	0.8	<0.1			2				2
29	2	B	HO/HE	1	0.1	2.9			2				2
32	2	B	HO	<1	<0.1	<0.1			2				2
33	2	B	HE	<1	0.2	1.0			2				2
37	2	B	HO	<1	1.2	1.9			2				2
40	2	B	HO	2	2.6	1.9			2				2
43	2	B	HO	<1	<0.1	<0.1			2				2
44	2	B	HO	<1	0.8	7.7			2				2
45	2	B	HO	4	0.5	1.0			2				2
7B	2	01_AE	HE	<1	1.0	2.0			2		2		
8B	2	F	HE	3	0.2	1.0			2				2
9B	2	C	HE	2	0.8	5.2			2				2
10B	2	02_AG	HE	4	0.8	5.3			2				2
13B	2	UD	HE	<1	<0.1	<0.1			2				2
17B	2	A	HE	<1	0.5	<0.1			2				2
18B	2	01_AE	HE	<1	1.0	2.0			2				2
20B	2	BF	HE	<1	0.1	<0.1			2				2
21B	2	02_AG	HE	1	0.6	5.3			2				2
23B	2	01_AE	HE	<1	0.6	1.0			2				2
Mixed clusters (n=18)													
10	11	B	HO	7	1.3	12.4	4		7				11
2B	8	A	IVDU	7	1.9	10.0		1	7				8
28	7	B	HO	3	0.9	10.6		1	5	1			7
1a	5	B	HE	4	0.8	9.0	1	1	3				5
14	5	B	HO	4	1.6	10.5	2		2	1			5
35	5	B	HO	2	0.7	4.3	1		4				5
38	5	B	HO	6	3.5	6.8	2	1	2			2	3
34	4	B	HO	1	1.1	1.8	1	1	2				4
9	3	B	HO	2	1.6	1.0	1	1	1				3

Table 3 continued.

Cluster ID	Patients, No.	Viral Subtype	Main transmission mode	Interval between first and last diagnosis, y	Genetic distance, mean, substitutions/site, %		GTD per FPR category, No. of patients				PTD, No. of patients		
					PR+RT	V3	≤5.75%	>5.75% – ≤10%	>10%	NA	R5	X4/DM	NA
16	3	B	HO	2	0.4	1.0		1	2				3
23	3	B	HO	4	1.6	7.4	1	1	1				3
1B	3	01_AE	HE	1	0.2	1.0	1		2		3		
8	2	B	HO	6	1.2	4.1		1	1				2
21	2	B	HO	3	0.5	3.1	1		1				2
36	2	B	IVDU	1	0.5	1.0	1		1				2
46	2	B	HO	2	2.4	0.9		1	1				2
3B	2	01_AE	HE	<1	0.5	<0.1	1		1		2		
16B	2	F	HO	1	0.5	1.0	1		1				2
X4 clusters (n=7)													
42	5	B	HO	1	0.2	<0.1	5					5	
5B	5	02_AG	HO	7	3.3	2.4	2	3			5		
27	2	B	IVDU	2	0.4	3.9	2					2	
30	2	B	HO	<1	0.2	<0.1	2				2		
48	2	B	HO	1	0.7	0.9	2					2	
14B	2	01_AE	HE	<1	0.1	<0.1	2					2	
15B	2	01_AE	HE	<1	0.1	1.1	1	1			1	1	

Data include number of individuals in each cluster, subtype, transmission route of the majority of individuals in the cluster, time interval between diagnosis in the earliest and latest cluster members, average genetic distance within the *pol* gene and the V3 fragment per cluster, number of members in each FPR category, and results of the phenotypic tropism analysis, if available.

Abbreviations: DM, dual/mixed; FPR, false-positive rate; HE, heterosexual contact; HO, homosexual contact; NA, not available; PR, protease; RT, reverse transcriptase; UD, undefined; IVDU, intravenous drug use; GTD, Genotypic tropism determination; PTD, Phenotypic tropism determination.

DISCUSSION

Studies on HIV-1 coreceptor use in treatment-naive individuals reported CXCR4 use in 4%–38% [12, 15, 29–35]. This high variability may be influenced by different compositions of the studied populations as well as by differences in methodology. Findings are based on results of phenotypic or genotypic assays for coreceptor determination, and although the latter all rely on V3 population sequencing, the algorithms used to deduce the coreceptor tropism vary. Geno2pheno is one of the prediction algorithms most extensively evaluated and is gaining wide acceptance in routine clinical practice in Europe as a tool to define susceptibility to the CCR5 antagonist maraviroc [4, 26]. One feature of Geno2pheno is the possibility of selecting the interpretative cutoff or FPR. The higher the FPR, the greater the likelihood of detecting CXCR4-using virus, but also the greater the likelihood of falsely declaring a sequence X4. Although there is currently no consensus on the FPR, clinical evidence provides support for the validity of using an FPR between 5% and 10% [25, 26, 36, 37]. We based our analysis on 2 cutoffs, 10% and 5.75%. For a population of 539 patients with newly diagnosed infection, this resulted in 19% and 12% X4/DM interpretations, respectively. Whatever the cutoff used, low baseline CD4 count and infection with a CRF01_AE virus came out as predictive for CXCR4 use. The association between CXCR4 use and low CD4 count is known [2, 32, 35] and was confirmed, but the worse immunologic status was not reflected in higher viral load. Lack of association between viral load and coreceptor use has been reported before [15, 29, 33, 38], though others saw the reverse [32]. The latter study did not select for recent diagnoses, so the contradictory findings may be caused by differences in study population and may suggest that higher viral loads are a secondary effect that becomes apparent after the drop in CD4 cells.

We observed a borderline correlation ($P = .05$) between infection through IVDU and X4/DM viruses, confirming previous findings [15, 29, 33]. With regard to homosexual or heterosexual transmission, no difference in coreceptor use was seen, and this is in line with other reports [15, 29, 33, 35]. Higher prevalence of DRMs was found in X4/DM viruses compared with R5 viruses but only when the 10% FPR cutoff was applied. In their study on primary infections, Raymond et al and Frange et al did not observe a correlation between DRMs and coreceptor use [13, 29]. Again, differences in study population could account for this difference in findings, and additional research to address this particular issue is needed.

The possible association between a defective CCR5 gene and the presence of X4/DM variants is a controversial issue. It can be assumed that reduced cell-surface CCR5 availability caused

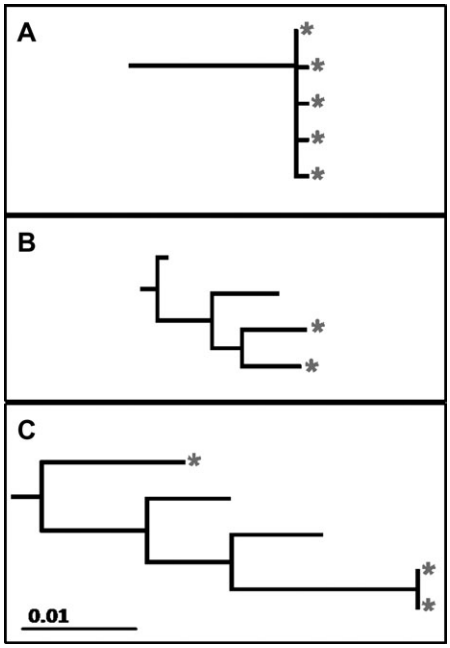

Figure 2. Representative transmission clusters. *A,* X4/DM cluster (cluster 42). *B, C,* Mixed clusters (clusters 34 and 38). Asterisks indicate X4/DM samples, and branch lengths reflect evolutionary distance.

by the Δ32 deletion favors X4/DM infection. This hypothesis was confirmed by findings of Brumme et al [35] but, because the patients tested had not have recently diagnosed infection, their data do not allow discrimination between higher susceptibility to X4/DM infection and faster evolution from CCR5 to CXCR4 use in the CCR5-heterozygous individuals. The inability by us and others to confirm the association reported by Brumme et al in individuals with recently diagnosed infection or seroconverters [18] favors the hypothesis of faster coreceptor switch, but more data are needed for confirmation.

Most studies that addressed HIV-1 coreceptor use were restricted to subtype B infections [13, 38], and those that included non-B subtypes either suffered from low sample numbers [15, 30, 39] or focused on one specific subtype, such as C [40–44] or 02_AG [45]. Although subtype C is believed by some to show an underrepresentation of X4/DM viruses [41, 43, 44], we and others before us have not been able to confirm this [30].

Our results, however, did reveal a significantly higher representation of viruses predicted as being able to use CXCR4 in CRF01_AE infections than in infections with subtype B, A, C, or CRF02_AG. There were no indications for a different ratio of acute versus chronic infections in the CRF01_AE population compared with the other subtypes (results not shown). Information on the reliability of Geno2pheno for the prediction of coreceptor use in non-B subtypes is still scarce, so we cannot exclude the possibility that our conclusions result in part from an overestimation in the prediction of CXCR4 use in CRF01_AE. Despite this possible bias, our findings do have important consequences and warrant further investigation. Because genotypic tropism prediction by Geno2pheno is now widely used in Europe to screen patients for their eligibility for maraviroc, the higher rate of CXCR4 prediction will result in a higher number of CRF01_AE-infected patients being deprived of this drug.

The report by Zhu et al in 1993 that new HIV-1 infections were nearly always initiated by macrophage tropic, non-syncytium-inducing variants led to the hypothesis of selective transmission favoring R5 strains [5]. This hypothesis was later fueled by the finding that individuals who genetically lack CCR5 expression are highly resistant to HIV-1 [6, 46, 47]. The mechanism behind this restriction at or after transmission remains unclear, and in vivo data to support the selection at transmission are scarce. We used data obtained through phylogenetic analysis of a region within the HIV-1 genome that is not involved in coreceptor use to allocate transmission clusters and analyzed the coreceptor use in these clusters. In 11% of all transmission clusters, genotypic tropism determination indicated the presence of virus able to use CXCR4 in each member of the clusters. We found indications for X4/DM transmission by IVDU, homosexual contact, and heterosexual contact, irrespective of the subtype.

Additional analysis in 13 documented transmission pairs showed CXCR4 use in 4 (31%). Three of the 4 documented X4/DM transmissions were confirmed by phenotyping, and in the 1 transmission pair in which discordant phenotypic results were obtained, the findings of limiting dilution analysis and the phenotypic data are highly suggestive for transmission of an X4 strain.

Exclusive use of CXCR4 is extremely rare in clinical samples [30] so the majority of viruses classified as X4/DM will be dual or mixed tropic, making it impossible to draw conclusions on the coreceptor used for initial infection. We were unable to show any evidence for selective transmission of R5 viruses, despite the observation of transmission clusters with R5 as well as X4/DM infections. The topologies of the mixed clusters as well as the higher genetic distances in V3 and *pol* support longer periods of independent evolution after transmission. It is therefore likely that X4/DM viruses in mixed clusters mainly result from posttransmission evolution.

Large epidemiologic cohort studies performed in the United Kingdom [48], Switzerland [49], and Quebec [50] demonstrated that early infection probably accounts for up to two-thirds of transmission events. Because the overall prevalence of X4/DM viruses in early infection fluctuates between 10% and 20%, the relative risk of exposure to X4/DM virus will be on this order of magnitude, which is supported by our findings. This warrants reconsideration of the general assumption that low prevalence of CXCR4-using viruses in recent infection indicates a transmission bottleneck.

Table 4. Characteristics of Documented Transmission Pairs and V3 Amino Acid Sequences

Patient IDs by pairs	Cluster	Viral Subtype	Genetic distance, substitutions/site, % PR+RT	Genetic distance, substitutions/site, % V3	Transmission route	PTD	GTD	FPR	Amino acids sequence V3 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
1A	39	B	0.1	<0.1	HO	R5	R5	89.1	C	T	R	P	N	N	N	T	R	K	G	I	H	I	G	P	G	R	T	F	F	A	T	G	A	I	I	G	N	I	R	Q	A	H	C
1B						R5	R5	89.1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
2A	39	B	<0.1	<0.1	HO	R5	R5	84.3	C	T	R	P	N	N	N	T	R	K	G	I	H	I	G	P	G	R	T	F	F	A	T	G	D	I	I	G	D	I	R	K	A	H	C
2B						R5	R5	84.3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
3A	39	B	0.1	<0.1	HO	NA	R5	84.3	C	T	R	P	N	N	N	T	R	K	G	I	H	I	G	P	G	R	T	F	F	A	T	G	D	I	I	G	D	I	R	K	A	H	C
3B							R5	84.3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
4A	25	B	0.1	<0.1	HO	NA	R5	59.2	C	T	R	P	N	N	N	T	R	K	S	I	H	I	G	P	G	R	A	F	Y	A	T	G	D	I	I	G	N	I	R	Q	A	H	C
4B							R5	59.2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
5A	32	B	<0.1	<0.1	HO	NA	R5	45.1	C	T	R	P	N	N	N	T	R	K	G	I	H	I	G	P	G	R	A	F	Y	A	T	G	D	I	I	G	D	I	R	Q	A	H	C
5B							R5	45.1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
6A	1B	01_AE	<0.1	<0.1	HE	R5	R5	10.5	C	T	R	P	S	N	N	T	R	T	S	I	A	I	G	P	G	Q	V	W	Y	R	T	G	E	I	I	G	D	I	R	K	A	Y	C
6B						R5	R5	10.5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
7A	13B	CRF 05_DF	<0.1	<0.1	HE	NA	R5	85.9	C	T	R	P	N/S	N	N	T	R	K	S	I	H	I/L	G	P	G	Q	A	F	Y	A	T	G	D	I	I	G	D	I	R	K	A	H	C
7B							R5	58.5	.	.	.	.	N	.	.	.	.	.	.	./P	.	.	.	.	.	.	./R	.	.	.	.	./D	./A	.	.	.	.	.	.	.	.	.	.	.
8A	17	B	0.1	<0.1	HO	NA	R5	25.5	C	T	R	P	N	N	N	T	R	R	S	I/V	H	I	G	P	G	K	A	F	Y	G	T	-	D	I	I	G	D	I	R	Q	A	H	C
8B							R5	21.5	.	.	.	.	.	.	.	.	./K	.	I	.	./M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
9A	3	B	0.1	0.9	HO	NA	R5	63.1	C	T	R	P	N	N	N	T	R	K	S	I	H	I	G	P	G	S	A	F	Y	A	T	G	D	I	I	G	D	I	R	Q	A	H	C
9B							R5	49	.	./I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
10A	14B	01_AE	0.1	<0.1	HE	X4	X4	0.2	C	T	R	P	F	K	L	E	K	K	M	T	S	-	G	P	G	H	V	F	Y	T	T	G	K	I	I	G	D	T	K	K	A	Y	C
10B						X4	X4	0.1	.	.	.	.	.	.	.	.	.	.	./K	.	.	.	.	.	.	.	.	.	.	.	.	./S	.	.	.	.	.	./R	.	.	.	.	.
11A	42	B	0.1	<0.1	HO	D/M	X4	3.8	C	T	R	P	G	N	N	T	R	K	S	I	H	L	G	P	G	R	A	W	Y	T	T	G	E	V	I	G	N	P	R	K	A	H	C
11B						D/M	X4	3.8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
12A	38	B	<0.1	<0.1	HO	D/M	X4	5	C	I	R	P	Y	N	N	T	R	R	S	I	H	I	G	P	G	R	A	Y	Y	A	P	G	E	I	I	G	D	I	R	K	A	H	C
12B						D/M	X4	4.7	.	.	.	.	./N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	./K	.	.	.	.	.	.	.	.	.	.	.
13A	15B	01_AE	0.1	14.7	HE	X4	X4	0.1	C	T	R	P	Y	N	S	R	R	A	K	T	A	I	G	Q	G	Q	V	F	Y	K	T	G	E	I	T	G	D	I	R	K	A	Y	C
13B						R5	X4	8.7	.	.	.	.	S	T	N	T	.	T	S	V	.	.	.	P	.	.	A	L/F/W	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.
13B-1			NA	NA		NA	X4	6.8	.	.	.	.	S	T	N	T	.	T	S	V	.	.	.	P	.	.	A	W	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.
13B-2			NA	NA		NA	X4	6.8	.	.	.	.	S	T	N	T	.	T	S	V	.	.	.	P	.	.	A	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.
13B-3			NA	NA		NA	X4	0	.	.	.	.	.	.	.	K	.	T	R	.	.	.	.	.	.	.	.	.	L	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.

Abbreviations: HE, heterosexual contact; HO, homosexual contact; GTD, Genotypic tropism determination; PTD, Phenotypic tropism determination; NA, not available.

The presence of X4/DM virus in acute infection can have clinical implications, because it has been associated with faster disease progression [29]. It might therefore be worthwhile to consider the possibility of early coreceptor tropism screening and early treatment of those individuals in whom X4/DM viruses are detected in order to prevent fast immune deterioration and halt the transmission of these strains.

Notes

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

Authors' contributions. K. C. designed and performed the experiments and the phylogenetic and statistical analysis and prepared the manuscript; K. D. and L. F. performed and validated the sequencing reactions; C. S. D. and F. B. were responsible for the phenotypic tropism analysis; B.V. was responsible for patient inclusion and clinical follow-up; L. V., J. P., and D. V. gave support and conceptual advice for the design of the study; C. V. developed the concept and supervised the study at all stages. All authors discussed the results and commented on the manuscript.

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