Characterization of Human Immunodeficiency Virus Type 1 (HIV-1) Diversity and Tropism in 145 Patients With Primary HIV-1 Infection

Philip Rieder,[1] Beda Joos,[1] Alexandra U. Scherrer,[1] Herbert Kuster,[1] Dominique Braun,[1] Christina Grube,[1] Barbara Niederöst,[1] Christine Leemann,[1] Sara Gianella,[1,a] Karin J. Metzner,[1] Jürg Böni,[2] Rainer Weber,[1] and Huldrych F. Günthard[1]

[1]Division of Infectious Diseases and Hospital Epidemiology, University Hospital Zurich; and [2]Swiss National Center for Retroviruses, Institute of Medical Virology, University of Zurich, Switzerland

(See the Editorial Commentary by Loes, on pages 1280–2.)

Background. In the context of sexual transmission of human immunodeficiency virus type 1 (HIV-1), current findings suggest that the mucosal barrier is the major site of viral selection, transforming the complex inoculum to a small, homogeneous founder virus population. We analyzed HIV-1 transmission in relation to viral and host characteristics within the Zurich primary HIV-1 infection study.

Methods. Clonal HIV-1 envelope sequences (on average 16 clones/patient) were isolated from the first available plasma samples during the early phase of infection from 145 patients with primary HIV-1 infection. Phylogenetic and tropism analyses were performed. Differences of viral diversities were investigated in association with several parameters potentially influencing HIV-1 transmission, eg, concomitant sexually transmitted infections (STIs) and mode of transmission.

Results. Median viral diversity within *env* C2-V3-C3 region was 0.39% (range 0.04%–3.23%). Viral diversity did not correlate with viral load, but it was slightly correlated with the duration of infection. Neither transmission mode, gender, nor STI predicted transmission of more heterogeneous founder virus populations that were found in 16 of 145 patients (11%; diversity >1%). Only 2 patients (1.4%) were assuredly infected with CXCR4-tropic HIV-1 within a R5/X4-tropic–mixed population, as revealed and confirmed using several genotypic prediction algorithms and phenotypic assays.

Conclusions. Our findings suggest that transmission of multiple HIV-1 variants might be a complex process that is not dependent on mucosal factors alone. CXCR4-tropic viruses can be sexually transmitted in rare instances, but their clinical relevance remains to be determined.

The extensive genetic diversity of human immunodeficiency virus type 1 (HIV-1) is a tremendous challenge regarding the development of broadly effective vaccines and also for antiretroviral treatment. The genetic bottleneck during HIV-1 transmission may be an Achilles heel of HIV-1 [1]. The mucosal barrier could be a key factor in driving the genetically complex viral inoculum to a homogeneous founder population. The impact of the mucosal barrier may depend on the anatomy, physiology, concurrent sexual transmitted infections (STIs), and sexual practice [2–4]. However, a genetic bottleneck has also been observed in intravenous drug users (IVDU), indicating mechanisms independent of the mucosa [3, 5].

During transmission, there is also strong selection for viral variants using CCR5 as a coreceptor [6, 7]. In-depth characterization of HIV-1 tropism in larger groups of patients during primary HIV-1 infection

(PHI) is needed to revisit recent work reporting varying frequencies of CXCR4-tropic strains during PHI [8–10]. Such data are of particular importance, because CCR5 antagonists are approved for clinical use mainly in salvage treatment [11, 12].

Here, we analyzed the complexity of virus populations within the C2-V3-C3 region of the HIV-1 envelope in 145 acutely and recently infected Zurich primary HIV-1 infection (ZPHI)-study patients [13–17] belonging to different transmission groups and infected with various viral subtypes. We investigated viral and host characteristics associated with elevated viral diversity and coreceptor tropism.

MATERIALS AND METHODS

Patient Characteristics

Patients were enrolled in the ZPHI study (http://clinicaltrials.gov, ID = NCT00537966) [13–17]. Acute/recent PHI was confirmed in all patients according to previously published definitions [15]. We estimated a date of infection for each patient by integrating all available clinical and laboratory data [15, 18]. During the first visit, each patient was checked for symptoms and physical signs of concurrent STIs and serology for syphilis. When dysuria, genital ulcers, groin, and/or rectal pain was present, urine and/or rectal swabs were subjected to polymerase chain reaction (PCR) analysis for *Neisseria gonorrhoeae*, *Chlamydia trachomatis* (when positive, specific PCR for lymphogranuloma venereum associated L-serovars was performed), and Herpes simplex.

Sequencing

RNA extraction, amplification, cloning, and sequencing of HIV-1 *env* C2-V3-C3 fragments were performed according to Rieder et al [15] by modification of previously described methods [19, 20].

Genotypic Prediction of HIV-1 Coreceptor Usage

V3-loop sequences were interpreted by three different genotypic prediction tools: 1) Web-PSSM http://indra.mullins.microbiol.washington.edu/webpssm/), 2) Wetcat http://genomiac2.ucsd.edu:8080/wetcat/v3.html), and 3) geno2phen[coreceptor] [7] (http://coreceptor.bioinf.mpi-inf.mpg.de/index.php). We used geno2pheno[coreceptor] with a false-positive rate of 5.75% [21]. Wetcat analysis is based on support vector machine (SVM) classifier. Position-specific scoring matrices (PSSM) predictions were performed by HIV-1 subtype B and C SINSI matrices for all non-C subtypes and clade C, respectively.

Phenotyping Assays

The cell lines MT-2 (D Richman, University of California, San Diego [22]), GHOST Parental Cell Line, and GHOST Cell Transfectants GHOST CXCR4 and Hi-5 (VN KewalRamani and DR Littman, New York, NY [23]) were obtained from the AIDS Research and Reference Reagent Program (Division of AIDS,

National Institute of Allergy and Infectious Diseases, National Institutes of Health). Syncytium-inducing phenotypes were determined in MT-2 cell culture assays [24]. If available, primary isolates, derived from both plasma and peripheral blood mononuclear cells (PBMCs), were tested. In addition, GHOST cell lines expressing CCR5 or CXCR4 and CD4 or CD4 alone were used to determine coreceptor usage. Cells were infected with primary virus isolates of each patient at a multiplicity of infection of 0.06. Expression of green fluorescent protein under control of the HIV-2 long terminal repeat promoter was detected by fluorescence microscopy [25].

Phylogenetic Analyses

Sequences were edited with SeqMan-5.08 software (DNASTAR Inc., Madison, WI), aligned with MAFFT-6.240 [26, 27], manually corrected, and tested for hypermutations by Hypermut 2.0 www.hiv.lanl.gov). Molecular evolutionary analyses were conducted using MEGA-4 [28]. Neighbor-joining phylogenetic (NJ) trees were constructed by MEGA-4 as well as Seqboot, Dnadist, and "neighbor and consense" (PHYLIP-3.68, distributed by J Felsenstein, University of Washington, Seattle). The reference strain HIV-1$_{HXB2}$ (GenBank accession no. K03455) and other B and non-B strains were used as outgroup references and bootstrapping (1000 or 100 replications with MEGA-4 and PHYLIP-3.68, respectively). *Pol* sequences were obtained from the Swiss HIV Cohort study drug resistance database [29]. Pairwise distances were computed by using MEGA-4 [28]. Nucleotide diversities were obtained using the Tamura-Nei model. All reported sequences have been deposited in GenBank under accession numbers GU471280 to GU471390, GU471407 to GU471578, and JF958169 to JF960135.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism version 5 (GraphPad Software, San Diego, CA) and STATA 11 SE (StataCorp, College Station, TX). Nonparametric tests were used for group comparison (Mann-Whitney *U* test). Multiple linear regression was used to analyze the association of viral diversity with viral and host baseline characteristics. The following variables were considered in the model: Age, sex, transmission category, STI, acute retroviral syndrome (ARS), viral load, CD4-cell count, viral subtype, and estimated date of infection (EDI).

RESULTS

Patient Characteristics

We analyzed 145 patients comprising 131 males and 14 females who were enrolled in the ongoing ZPHI study (Table 1). The modes of transmission according to patients' statements included homosexual (73%), heterosexual (22%), bisexual (2%), IVDU (1.4%), and others (1.4%). Concomitant STIs have been

diagnosed in 20 patients (14%). HIV-1 subtype B was most prevalent (80%) followed by CRF01_AE (9%), C (3%), and A (3%). The remaining 5% of patients were infected with HIV-1 subtypes F1, G, CRF02_AG, and CFR12_BF.

One hundred twenty-five patients were diagnosed during documented acute HIV-1 infection. Of those, 120 patients (96%) had an ARS [13] and 87 (70%) had a negative or indeterminate Western blot (WB). Recent infection was diagnosed in 20 patients. Baseline blood samples were available within a median of 6 weeks (range 2–18) after EDI in acute and within 12 (range 7–24) weeks in recently infected patients. Fiebig staging [30] was possible for 117 patients: 2 patients were assigned to stage II, 14 patients to stage III, 52 patients to stage IV, 20 patients to stage V, 19 patients to stage V/VI, and 10 patients to stage VI.

Phylogenetic Reconstruction

NJ trees containing clonal C2-V3-C3 sequences from 113 (78%) patients formed individual clusters with bootstrap values of 100%. Clusters with bootstrap values <100% were found in 32 patients, most of whom also harbored closely related *pol* sequences (median genetic distance 0.0062; range 0–0.0124) except for 4 patients forming 2 clusters. Possible contamination was ruled out by analyzing an independent sample from each

of these 4 patients. Sequences from all patients showed no clustering with reference clones used in our laboratory.

Validity of Viral Diversity at Baseline

In total, 2268 clones (median 16 clones/patient; range 10–16) spanning C2-V3-C3 region of the *env* gene were derived from plasma HIV-1 RNA of 145 PHI patients early after transmission (median 6 weeks, range 2–24). The baseline viral diversities ranged from 0.04% to 3.23% (median 0.39%). To obtain representative samples of quasispecies, reverse transcription-PCR was performed in duplicate using HIV-1 RNA extracts from 1 ml of plasma. The viral load of the plasma samples ranged from 2.4 to 7.6 (median 5.3) $\log_{10}$ copies/ml. Despite this broad distribution, viral loads and nucleotide diversities were not correlated (Figure 1). The viral loads of 10 patients were <5000 copies/ml; however, viral diversities (median 0.44%; range 0.11–1.25%) were similar to patients with high viral loads ($P = .662$). Thus, the sequenced clones were representative of the actual plasma virus populations in vivo as previously shown in chronically infected patients [20]. In addition, single genome amplification ([SGA] median 12 sequences/sample) was performed in a subset of 25 patients, and the resulting viral diversities correlated well with clonal sequencing (slope = 1.05, intercept = –0.28%, $r^2 = 0.681$; data not shown). The viral

Table 1. Baseline Characteristics of 145 Patients With Primary HIV-1 Infection

|	Total patients		Female		Acute infection		Recent infection	
	n	%	n	%	n	%	n	%
Number of patients	145	100	14	100	125	100	20	100
Male	131	90			112	90	19	95
Female	14	10			13	10	1	5
HIV-1 subtype B[a]	116	80	5	36	99	79	17	85
Transmission mode								
Homosexual	106	73			91	73	15	75
Heterosexual	32	22	14	100	28	22	4	20
Bisexual	3	2			2	2	1	5
IVDU	2	1.4			2	2		
Others[b]	2	1.4			2	2		
STIs[c]	20	14	1	7	14	11	6	30
HIV-1 drug resistance transmitted[d]	6	4	1	7	6	5		
Acute retroviral syndrome	136	94	13	93	120	96	16	80
Negative or indeterminate Western Blot	87	60	6	43	87	70		
	median	(min-max)	median	(min-max)	median	(min-max)	median	(min-max)
Estimated duration of infection (weeks)	6	(2–24)	6	(2–14)	6	(2–18)	12	(7–24)
Viral load ($\log_{10}$ copies of HIV-1 RNA/ml plasma)	5.3	(2.4–7.6)	4.8	(4.2–6.7)	5.5	(2.4–7.6)	5.0	(3.2–6.5)
CD4+ T cells (cells/μl blood)	414	(87–1295)	454	(87–1295)	374	(127–1295)	503.5	(87–965)
Age (years)	36	(19–70)	32	(19–55)	37	(19–70)	34	(20–58)

Abbreviations: IVDU, intravenous drug users; STIs, sexually transmitted infections; HIV-1, human immunodeficiency virus type 1.

[a] Other subtypes: CRF01_AE, C, A, F1, G, CRF02_AG, CRF12_BF.

[b] One case needle stick; 1 case either IVDU or heterosexual.

[c] Concomitant STIs: syphilis and/or chlamydia and/or gonorrhea.

[d] International AIDS Society-USA mutation.
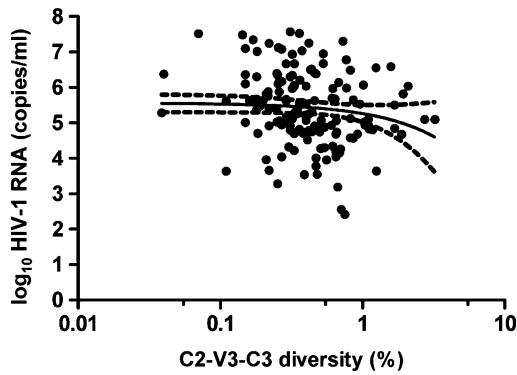
Figure 1. Relationship between plasma viral load and viral diversity during primary human immunodeficiency virus type 1 infection. Plasma viral load of the first available sample and the corresponding viral nucleotide sequence diversity observed in the C2-V3-C3 region of the envelope gene in 10–16 clones per patient is shown. One hundred forty-five patients were analyzed. Each dot represents 1 patient. The relationship between viral diversity and viral load is shown by linear regression (P = .109; slope = −0.299 ± 0.185; r^2 = 0.018).

diversities increased slightly with time since EDI (EDI was described in previously published methods [15, 18]) (Figure 2), supporting the reliability of the EDI calculation used. As also shown in Figure 2, negative WBs have been detected up to 10 weeks and positive WBs between 4 and 24 weeks after EDI, demonstrating the need to include clinical data into the diagnostic staging process.

Viral Diversity in Relation to Host and Viral Characteristics

In our study population, the median viral diversity shortly after transmission did not deviate among different sexual transmission categories (Figure 3A). Twenty patients, including 1 female, 5 men having sex with females, and 14 men having sex with men (MSM), were diagnosed with a concomitant STI, but their median viral diversity did not deviate from those without concomitant STI (P = .703; Figure 3B). Next, we analyzed in more detail the relation between diversity and transmission mode among MSM depending on the encountered mucosa. Analyzed sexual practices were insertive anal intercourse, receptive anal intercourse, and unsafe oral intercourse. Patients practicing various sexual activities or with unknown transmission route were excluded from this analysis. No significant differences among distinct transmission modes were observed (Figure 3C).

We also derived the viral subtypes from individual *pol* sequences to examine their possible effect on diversity (Figure 4). Four patients had been infected with HIV-1 subtype C. They showed a median viral diversity of 1.07% (range 0.54–1.89%), which significantly deviated from those observed in HIV-1 subtype B (P = .011). In addition, no association between viral diversity and viral tropism was found (for tropism see below). Moreover, multiple linear regression testing the association
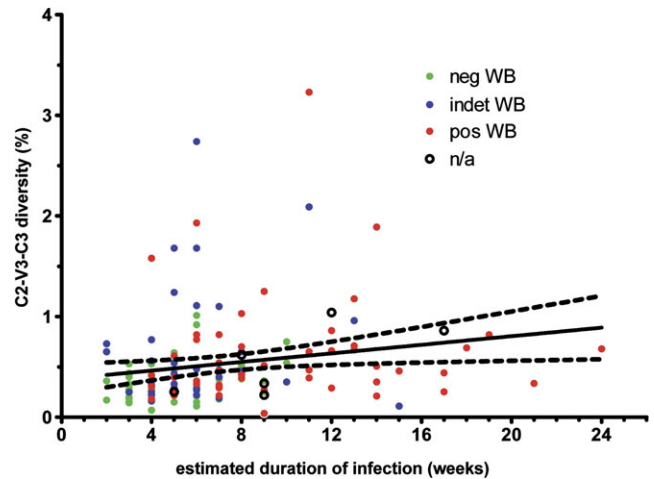

Figure 2. Relationship between viral diversity during primary human immunodeficiency virus type 1 (HIV-1) infection and estimated duration of infection. For each of the 145 patients, the duration between estimated date of infection (EDI) and first available sample and the corresponding viral diversity observed in the C2-V3-C3 region of the envelope gene in 10–16 clones is shown. The duration of infection has been estimated integrating all available information including patient history relating to known risk situations, occurrence of first symptoms, previous negative test results, avidity assays, and Western Blot (WB). The median viral diversities increased slightly with longer time intervals between infection and sample collection (slope = 0.024 ± 0.009; P = .015; r^2 = 0.041). Negative WBs were detected up to 10 weeks and positive WBs were detected between 4 and 24 weeks of EDI, respectively. Neg WB, negative WB; indet WB, indeterminate WB; pos WB, positive WB; n/a, not available.

of viral diversity with EDI, age, CD4-cell count, viral load, viral subtype, ARS, STI, and transmission mode showed no relationship except for EDI (slope = 0.031 [95% confidence interval = 0.001–0.061], P = .046, r^2 = 0.151).

Characteristics of Patients With High Viral Diversity

We identified 16 subjects (11%) with a viral diversity higher than 1% despite a short estimated duration of infection (median 7 weeks; range 4–14). Of the 14 males 3 had a concomitant STI. One of the heterosexual men was infected either by sexual contact or by sharing needles. For detailed patient characteristics see Table 2. Overall, no patient's or transmission's characteristics were overrepresented in the group of patients with viral diversity higher than 1%. These patients were infected with HIV-1 subtype A (1 of 4, 25%), B (12 of 116, 10%), and C (3 of 4; 75%).

Phylogenetic analyses of the C2-V3-C3 region in the 16 patients with viral diversities >1% showed 3 different tree patterns. In 4 patients, a starlike diversification was seen, whereas in 8 patients the viral clones appeared to be subdivided into 2 to 4 subclusters, each with diversity below 1% and average genetic distances between these intrapatient subgroups of 2.14% (range 1.11–4.37%). In the remaining 4 patients, a mixture of these 2
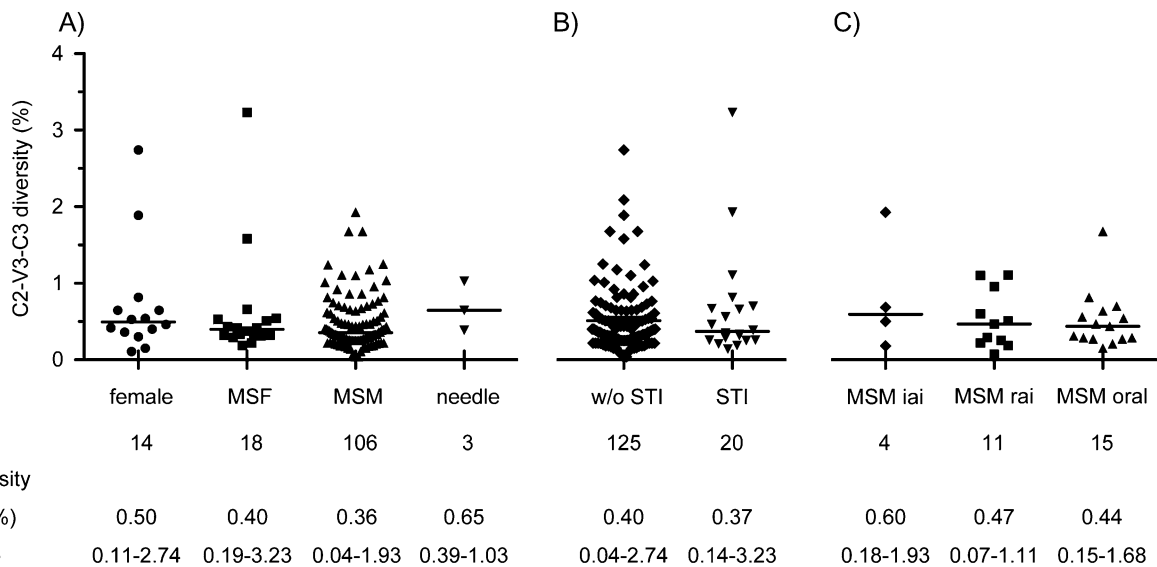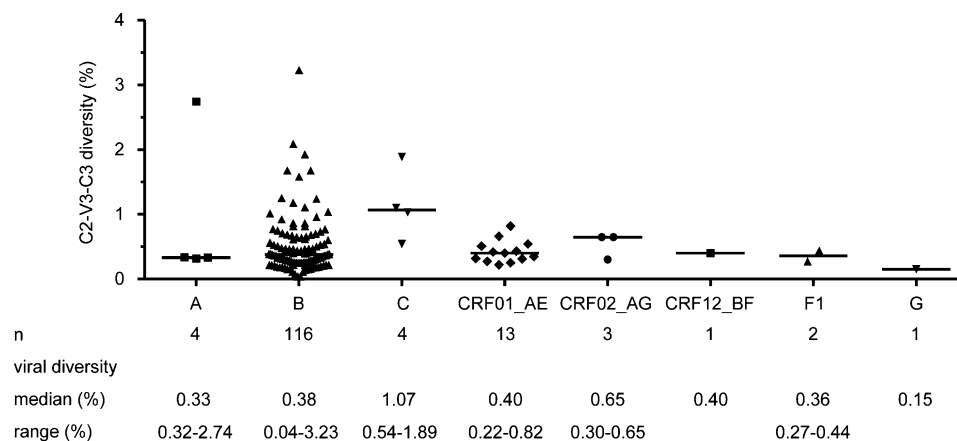
Figure 3. Influence of transmission characteristics on viral diversity during primary human immunodeficiency virus type 1 (HIV-1) infection. Viral diversities observed in the C2-V3-C3 region of the envelope gene in 144 primary HIV-1–infected patients are shown grouped according to gender and transmission category in *A* (1 patient infected by either heterosexual intercourse or needle sharing is not shown), according to the presence or absence of a concomitant sexual transmitted infection in *B*, and within the transmission category men having sex with men (MSM) according to the sexual practice during HIV-1 acquisition in *C*. In the latter analysis, MSM patients with unknown transmission mode or practicing several sexual behaviors were excluded from this analysis. Bars indicate medians; MSF, men having sex with females; w/o STI, without concomitant sexually transmitted infections; STI, with sexually transmitted infections; iai, insertive anal intercourse; rai, receptive anal intercourse; oral, unsafe oral intercourse.

described patterns was observed including subgroups showing viral diversity of more than 1%.

Viral Tropism

Using PSSM, SVM$_{wetcat}$, and geno2pheno[coreceptor] to predict viral tropism in clonal C2-V3-C3 sequences, X4-tropic viruses or R5/X4 mixed populations were predicted for 10%, 19%, and 14% of the patients, respectively (Table 3). In 102 patients (70%), all three prediction algorithms were consistent in predicting R5-tropic viral variants. The three bioinformatic tools

yielded conflicting coreceptor usage predictions in 27% of the patients. In 4 patients (3%), all three tools predicted X4-tropic strains, either as a mixed population in 3 cases or as a pure X4-tropic population in 1 case. Full-length *env* sequences obtained by SGA showed the same quasispecies composition and mutation patterns as clonal C2-V3-C3 sequences in these 4 patients (data not shown). Next, HIV-1 coreceptor usage was determined by phenotypic assays using 175 available primary isolates (from 117 patients) derived from plasma and PBMCs (Table 4). In the first step, all isolates were screened by the MT-2

Figure 4. Influence of HIV-1 subtypes on viral diversity during primary human immunodeficiency virus type 1 (HIV-1) infection. Viral diversities observed in the C2-V3-C3 region of the envelope gene in 145 primary HIV-1–infected patients are shown grouped according to HIV-1 subtypes. Bars indicate medians; CRF, circulating recombinant form.

Table 2. Baseline Characteristics of Patients With Higher and Lower Viral Diversity During Primary HIV-1 Infection

Viral diversity after transmission	>1%		<1%		
	n	%	n	%	
Number of patients	16	100	129	100	
Male	14	88	117	91	
Female	2	13	12	9	
HIV-1 subtype					
Subtype A	1	6	3	2	
Subtype B	12	75	104	81	
Subtype C	3	19	1	1	
CRF01 _AE			13	10	
Transmission mode					
Homosexual	10	63	96	74	
Heterosexual	3	19	29	22	
STIs[a]	3	19	17	13	
Negative or indeterminate Western Blot	9	56	78	60	
	median	(min-max)	median	(min-max)	P value
Estimated duration of infection (weeks)	7	(4–14)	6	(2–24)	.127[b]
Viral load (log$_{10}$ copies of HIV-1 RNA/ml plasma)	5.1	(3.6–6.6)	5.4	(2.4–7.6)	.623[b]
CD4$^+$ T cells (cells/μl blood)	483	(127–1295)	411	(87–1120)	.643[b]
Age (years)	32	(19–60)	36	(20–70)	.403[b]

Abbreviations: HIV-1, human immunodeficiency virus type 1; STIs, sexually transmitted infections.

[a] Concomitant sexually transmitted infections: syphilis and/or chlamydia and/or gonorrhea.

[b] Mann-Whitney *U* test.

cell assay and if X4-tropism was detected, this was confirmed by the GHOST cell assay, allowing also differentiation of mixtures of R5/X4 double users, respectively. In 1 of the 4 patients with concordant genotypic X4-tropism prediction and in 1 patient with concordant CCR5-tropism prediction, a mixture of R5/X4-tropic viruses was detected in the PBMC isolate but not in the isolate obtained from plasma.

Table 3. Genotypic Prediction of HIV-1 Tropism in Patients With Primary HIV-1 Infection

HIV-1 Subtypes	All		B	
	n	%	n	%
Number of patients	145	100	114	100
X4 tropism prediction by:[a]				
Geno2pheno[coreceptor][b]	21	14	13	11
PSSM[c]	15	10	5	4
SVMwetcat	28	19	24	21
1 of 3 tools predicts X4 tropism	26	18	21	18
2 of 3 tools predict X4 tropism	13	9	6	5
3 of 3 tools predict X4 tropism	4	3	3	3
R5 tropism prediction by 3 tools	102	70	84	74

[a] X4 or R5/X4 mixed populations.

[b] Cutoff 5.75 (Prosperi et al. Retrovirology 2010, 7:56).

[c] Using sinsi matrix.

DISCUSSION

To investigate the complexity of transmitted HIV-1 populations, we determined viral diversity by HIV-1 envelope C2-V3-C3 clonal sequencing and assessed potential factors associated with transmission of heterogeneous viral populations in 145 patients enrolled in a single center primary HIV-1 infection cohort (ZPHI study). This analysis revealed three major findings: 1) heterogeneous virus populations (diversity >1%) were found in 11% of all patients; 2) neither concomitant STI, gender, nor differences in sexual practices could be identified as factors associated with transmission of heterogeneous virus populations; and 3) genotypic prediction of coreceptor tropism by 3 computational tools unambiguously characterized transmitted viruses of 73% of all patients. Seventy percent of patients harbored R5-tropic viruses, and 3% (4 patients) were predicted as harboring X4-tropic and R5/X4-tropic viruses, respectively. However, in only 1 of those, CXCR4-usage could be confirmed phenotypically. In addition, 1 patient consistently predicted as harboring R5-tropic viruses, showed phenotypically mixed R5/X4-tropic viruses. Furthermore, results remained genotypically ambiguous in 27%.

Despite various efforts [2–5, 31, 32], factors associated with increased viral heterogeneity, which is found in ∼10%–20% of PHI patients, have not been fully elucidated. However, this

Table 4. Phenotypic Prediction of HIV-1 Tropism of Primary HIV-1 Isolates Obtained From Plasma and PBMCs of Primary HIV-1–Infected Patients

	Genotype[a]	Phenotype			
	Number of patients predicted by 3 algorithms	Number of patients analyzed by MT-2 assay	X4	R5	R5/X4
X4	4	4	0	3 (75%)	1 (25%)[b]
R5	102	83	0	82 (98.8%)	1 (1.2%)[c]
inconsistent	39	30	0	30 (100%)	0

[a] Predicted by 3 bioinformatic tools: Geno2pheno, PSSM, and SVMwetcat.
[b] Detected in peripheral blood mononuclear cells (PBMCs) with MT-2 and GHOST cell assay.
[c] Detected in PBMCs with MT-2 but not with GHOST cell assay.

result is not surprising given the difficulties to identify large populations of well-characterized patients with PHI and the multifactorial nature affecting diversity upon transmission. In particular, interpretation of results may be complicated by, 1) differences in patient populations with regard to time of sampling after infection; 2) disparities in ethnicity and living environments such as access to health care, sanitation conditions, and nutritional status; 3) potential selection bias of patients enrolled in PHI studies; 4) uncertainties concerning sexual practices adopted; 5) the lack of knowledge of potential host genetic factors; and 6) different methodologies used to investigate early transmitted virus.

Our finding of complex HIV-1 populations in 11% of patients is in line with previous findings [2], although at a lower range. A likely explanation for these differences might be different frequencies of index patients transmitting a homogeneous virus population while they are in the acute phase of their HIV-1 infection. Another potential explanation is our rather conservative approach, compared with others [1], to search for complex viral populations using a cutoff of 1% diversity to differentiate homogeneous from heterogeneous transmitted viruses.

In contrast to previous studies [3, 31–33], no elevated complexity of transmitted viruses was found in the 20 patients with a concomitant STI during PHI. STIs clearly increase the risk of HIV-1 transmission by enhancing infectiousness and susceptibility [34]. The lower viral diversity in our patients might be due to the time of the transmission of the STI. Ongoing and already established STIs prior to HIV-1 infection may be of higher importance to render the mucosal barrier more susceptible for HIV-1 infection than those transmitted concurrently with HIV-1 or shortly thereafter. In our setting, the vast majority of patients reported first occurrence of STI symptoms very closely to ARS symptoms, suggesting that STIs were cotransmitted with HIV-1. In contrast, Haaland et al [31] reported that their Zambian and Rwandan seroconverter patients had symptoms and signs of STIs that were already present at the time of the last seronegative visit, suggesting that those STIs were present for comparatively longer durations, and therefore, mucosa may have been damaged more severely at the time of HIV-1 infection. Another difference between cohorts may be the proportion of female sex workers included [4]. In this group, the prevalence of STIs and the occurrence of mucosal microtrauma are higher than those in males or in females in general [3, 4, 35]. Female sex workers did not participate in our study.

HIV-1 subtype C may be associated with greater propensity for transmission possibility than other HIV-1 group M subtypes [34]. In our study, the 4 patients infected with HIV-1 subtype C showed a higher median viral diversity compared with HIV-1 subtype B-infected patients ($P = .011$). Viral diversity of CRF01_AE and other circulating recombinant forms were not significantly lower compared with HIV-1 subtype B, but none of those patients was infected with a more complex population (>1%). Whether higher diversity seen in our HIV-1 subtype C cases is based on HIV-1 subtype-specific viral properties or not cannot be proven at this time. Similar diversities found in HIV-1 subtypes B and C in other PHI cohorts question the biological relevance of our finding [33]. However, it has to be noted that comparisons between those 2 HIV-1 subtypes were based on different study settings in different countries and at different centers [33]. Thus, ultimately, only studies of larger patient cohorts enrolled under similar conditions will yield an answer to this question.

By combining 3 different genotypic prediction algorithms, we found that 4 PHI patients (3%) possibly harbored CXCR4-tropic viruses during acute HIV-1 infection. However, in only 1 of those patients, X4-tropic strains could be confirmed phenotypically. In addition, from 1 patient with consistent R5-coreceptor prediction, X4-tropic strains could be isolated within a R5/X4 mixture. Thus, in our study, transmission of relevant replication competent X4 variants seemed to be a rare event. These findings are in line with Raymond et al [8] who reported 6.4% of plasma viruses as X4-tropic and R5/X4-tropic mixtures, respectively, in patients with acute HIV-1 infection. In contrast, a recent study using ultra-deep sequencing and genotypic prediction reported X4-tropic variants in ~50% of a small number of patients with acute HIV-1 infection [9]. Discrepant results are not surprising given the methodological difficulties regarding assays, prediction algorithms used, studied populations, and sample sizes. At present, the clinical relevance of transmitted X4-tropic minority variants is not known, because thresholds for frequencies of X4-tropic minority variants to predict maraviroc response during PHI are not available [36]. Furthermore, the problem with genotypic prediction is that it cannot predict replication competence of these minority species. On the other hand, phenotypic assays using primary viral isolates overcome this limitation but may lack sensitivity due

to initial selection process during their propagation in cell cultures [37]. Recombinant phenotypic assays using PCR-amplified HIV-1 envelope may have an improved sensitivity profile, but they give only information on tropism of single envelopes amplified and not on replication competence of these viruses. Taken together, despite a considerable amount of previous work [8–10, 38, 39], frequency of clinically relevant transmitted CXCR4-using viruses is still not known, but in our study transmission of such viruses seemed to occur only rarely.

In summary, our findings suggest that transmission of complex virus founder populations may not depend solely on mucosal factors. In addition, transmission of clinically relevant CXCR4-using virus strains seems to remain a rare event, and caution is warranted when predicting coreceptor usage by genotypic algorithms alone, because concordance of those tools is still limited.

Notes

Author contributions. Study concept, design, supervision: Günthard
Acquisition of laboratory data: Rieder, Joos, Böni, Leemann, Niederöst, Kuster, and Metzner
Acquisition of clinical data: Grube, Braun, Scherrer, Rieder, and Günthard
Database management: Scherrer, Braun, Gianella, and Günthard
Analysis and manuscript: Rieder, Joos, Scherrer, Metzner, and Günthard

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