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Construction and tropism characterisation of recombinant viruses exhibiting HIV-1 *env* gene from seminal strains

Philip Lawrence [a], Willy Berlier [a], Olivier Delezay [a], Sabine Palle [b], Thomas Olivier [b], Henia Saoudin [a], Stéphane Mottin [b], Frédéric Lucht [a,c], Bruno Pozzetto [a,*], Thomas Bourlet [a]

[a] Groupe Immunité des Muqueuses et Agents Pathogènes (GIMAP-EA 3064), Faculté de Médecine J. Lisfranc, Université Jean Monnet, IFRESIS et CHU de Saint-Etienne, 15 rue Ambroise Paré, 42023 Saint-Etienne cedex 02, France
[b] Centre de Microscopie Confocale Multiphotonique, Université Jean Monnet, Pôle Optique et Vision, Bâtiment F, 18 rue Professeur Benoît Lauras, 42000 Saint-Etienne, France
[c] Department of Infectious Diseases, CHU de Saint-Etienne, 25 bd Pasteur, 42100 Saint-Etienne, France

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ABSTRACT

Genetic differences between blood and mucosal-derived HIV-1 strains have been widely reported. As amplification of HIV-1 strains from mucosal samples including semen or saliva by co-culture has low sensitivity, we developed the construction of chimeric viruses expressing wild-type seminal HIV-1 envelope protein. Chimeric viruses were produced by co-transfection of a V1–V3 deleted pNL 43 vector and PCR fragments spanning the deleted region, amplified from HIV-1 RNA positive seminal plasma samples. After an initial testing of co-receptor usage by a tropism recombinant test, replication capacity and amplification of these recombinant viruses were assessed using PBMC. Four chimeric replicative strains, all using CXCR4 as coreceptor, were produced. The interaction between cell-free viral particles and reporter cell lines was assessed by confocal microscopy. These replicative chimeras exhibiting HIV-1 *env* from seminal strains represent useful tools for the *in vitro* study of the heterosexual transmission of HIV-1 and testing of microbicide activity.

Introduction

It is now well established that the entry of the human immunodeficiency virus type 1 (HIV-1) into permissive cells relies on an interaction between viral and cellular surface proteins. The binding of the viral envelope glycoprotein gp120 to the cellular CD4 protein induces conformational changes in the former that reveal a binding site for a cellular co-receptor (Sattentau and Moore, 1991; Wu et al., 1996), principally via CXCR4 or CCR5 molecules (Philpott, 2003; Simmons et al., 2000). It has been documented that a region spanning the variable regions 1 to 3 of the gp120 (V1–V3) is largely responsible for the interaction between the viral particle and its target cellular receptors and that the amino acid composition of this region, most notably of the V3 loop, can orientate the specific ability of a virus to use one or the other co-receptor (Koning et al., 2002).

The predominant mode of global HIV transmission is through the exposure of mucosal surfaces to male genital secretions carrying the virus. Mucosa represents not only the primary route of entry for the virus but also the initial and predominant site of viral replication and amplification. Evidence has shown that the local mucosal environment may influence viral replication, shedding and/or ultimately transmission (Brenchley et al., 2004; Veazey et al., 1998). In each chronically infected individual, HIV exists as quasispecies of related genetic variants and anatomic compartmentalization of these variants has been described for blood, the central nervous system, the genital tract, rectal mucosa, lung and saliva (Di Stefano et al., 2001; Gunthard et al., 2001; Gupta et al., 2000; Kemal et al., 2003; Kiessling, 1992; Liuzzi et al., 1996; Paranjpe et al., 2002; Singh et al., 1999; Zhang et al., 2002).

Genetic differences between blood and semen-derived HIV-1 have been widely reported (Byrn and Kiessling, 1998; Delwart et al., 1998; Gupta et al., 2000; Vernazza et al., 1997; Zhu et al., 1996), due at least in part to the fact that male genital tract tissues can serve as distinct sites of replication leading to strains exhibiting specific characteristics (Kiessling et al., 1998; Paranjpe et al., 2002; Ping et al., 2000). Recent studies suggest that the male genital tract represents a selective reservoir that leads to genetic bottlenecks associated with sexual transmission of HIV-1 (Pillai et al., 2005). HIV isolates of mucosal origin, either free virus or cell-associated, are seldom used in *in vitro* studies of sexual transmission even though they are probably more representative and pertinent than adapted laboratory strains. This is partly because of the fact that the amplification of HIV-1 strains from semen samples by PBMC culture is fastidious and has low sensitivity due to low virus loads and semen toxicity towards target cells (Coombs et al., 1998; Vernazza et al., 1997).

* Corresponding author. Fax: +33 4 77 42 14 86.
E-mail address: bruno.pozzetto@univ-st-etienne.fr (B. Pozzetto).

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Table 1
Clinical data of the 11 patients included in the study and comparison of V3 sequences from the seminal recombinant strains

Strain		Clinical data			Amino acids of the V3 loop[e]																																							Deduced tropism	
Number[a]	Sub-type	Clinical stage[b]	Viral load[c]	HAART[d]																																								Geno[f]	Pheno[g]
LAI	B	–	–	–	C	T	R	P	–	N	N	N	T	R	K	S	I	R	I	Q	R	G	P	G	R	A	F	V	T	I	G	K	I	–	G	N	M	R	Q	A	H	C		
BAL	B	–	–	–														H		–	–						L	Y		T				I		D	I		E				R5	R5
594	B	C3	4,5	Yes		I								I		G		H		–	–							Y		T		Q		V		D	I						R5	X4
609	B	A2	4,1	Yes														H		–	–							Y		–		D		I		D	I					Y	R5	R5
611	CRF02_AG	A2	6.0	No										I		G		H		–	–							Y		T		Q		V		D	I						R5	R5
611,6									T	H				I		G		H		–	–							Y		T		Q		V		D	I						R5	R5
611,7														I		G		H		–	–							Y		A		Q		V		D	I						R5	R5
611,9												I		I		G		H		–	–							Y		T		Q		V		D	I						R5	R5
654	B	A3	6.0	Yes						S	I		K		R	H		H		–	–							Y	A	T	E	R	N	–			I						X4	R5
654,12																G		H		–	–							Y		T		Q		V		D	I						R5	R5
662	B	A3	3.5	Yes														H		–	–				Q	T		Y	A	T		D		I		D	I						R5	R5
705	B	B2	3.8	Yes						S				G				P		–	–							H		–		R		I		D	I						X4	X4
728	B	B1	4.3	Yes											R	G		H	M	–	–		M					Y	A	T		R		I		D	I					Y	X4	X4
802	B	C3	3.1	Yes														P		–	–							Y		T		D		I		D	I						R5	R5
803	CRF02_AG	C3	6.0	No							R	K						P		–	–											D		I									X4	X4
804	B	B3	3.6	Yes						S	R	K	L	V	R	H		H		–	–							Y		T		R	E	T		K	I						X4	X4
805	CRF02_AG	C3	5.3	No													V			–	–				Q			Y	A	T		D		I			I						R5	R5

[a] Patients' chimeric strains are designated by numbers. For strains 611 and 654, different clones were analyzed (see text).
[b] Clinical stage determined according to CDC classification system for HIV infection.
[c] Viral load in blood expressed in log10 copies/ml.
[d] Highly active antiretroviral treatment.
[e] Alignment obtained with HIV-1 strain NL4-3 using CINEMA 5 software. Blanks indicate a match with the reference sequence whereas substitutions are indicated by the single letter code for the changed amino acid. Gaps are shown as dashes.
[f] Determined by using the algorithm available on http://www.geno2pheno.org/cgi-bin/geno2pheno.pl.
[g] Determined by the tropism recombinant test using U373-X4 or -R5 reporter cells.

In order to overcome the difficulties inherent in the isolation of cell-free or cell-associated seminal strains, we adapted a technique originally developed to test the tropism of viral strains using the V1–V3 deleted vector pNL 43 (Trouplin et al., 2001) for the construction of chimeric viruses expressing the V1–V3 region of envelope protein from wild-type seminal HIV-1 strains. Using this method, we obtained four replicating seminal-derived strains characterised for their CXCR4 tropism and able to interact as cell-free particles with a reporter cell line, as demonstrated by confocal microscopy.

Results

Amplification of V1–V3 from seminal and blood strains

Fragments of 940 bp containing the envelope region spanning V1–V3 of HIV-1 were successfully amplified by nested-PCR from 11 seminal plasma samples from HIV-infected subjects and, as controls, from the laboratory strains HIV-1_{LAI} and HIV-1_{BAL}. The clinical data of these 11 subjects are given in Table 1. For 6 of the 11 positive seminal samples, the V1–V3 region was also amplified by PCR from corresponding blood samples available at the same time. The V3 region was sequenced in order to compare blood and plasma strains. Differences were observed for 4 of the samples tested, with between 1 and 4 amino acid changes in the V3 loop.

Construction and growth characteristics of chimeric viruses

After co-transfection of bulk V1–V3 fragments together with the 43 ΔV deleted vector in 293T cells, all supernatants tested positive by p24 assay (mean level 200 ng/ml, ranging from 156 to 224, data not shown). As expected, the chimeric LAI and BAL controls gave positive signals on CXCR4+ and CCR5+ cells, respectively (data not shown). Testing of the above supernatants by the tropism recombinant test (TRT) described by Trouplin et al. (2001) gave a positive signal on CXCR4+ cells for two supernatants (samples 609 and 728). None of the supernatants was found positive with the CCR5+ cells.

In order to assess the cultivability of the chimeric viruses potentially generated by transfection experiments, all of the 11 supernatants and chimeric LAI and BAL controls were cultured with PHA/IL-2 activated PBMC for 16 days and quantified daily by p24 assay. Four strains (594, 705, 728 and 804) were found positive (data not shown). These strains also tested positive on CXCR4+ cells by the TRT, indicating the presence of PBMC-amplified X4 strains (Fig. 1). Interestingly, the lack of positivity for sample 609 after culture with PBMC (Fig. 1) despite a positive signal with the X4-TRT at day 0 and the detection of proviral DNA (data not shown) suggests that this chimeric virus was able to exhibit only one cycle of replication.

To investigate further the seminal samples from six patients found negative by the TRT (611, 654, 662, 802, 803 and 805), mixes of corresponding initial V1–V3 PCR products were each sub-cloned. Between 15 and 20 sub-clones were used to produce chimeric viruses. Testing of these supernatants by the TRT gave a positive signal on CCR5+ cells for two samples (611 — 4 distinct profiles and 654 — 2 profiles). Some of these clones were shown to present 1 to 3 amino acid differences when compared to the predominant profile; interestingly, one clone from sample 654 exhibited 12 amino acid changes. In order to assess the replicative capacity of these recombinant viruses, supernatants were cultured with PHA/IL-2 stimulated PBMCs and cultures were tested for p24 production daily for 16 days. No p24 production was observed for these viruses, despite positive proviral DNA detection, suggesting the presence of infectious, one-cycle particles (data not shown).

The four replicative chimeric strains characterised above and the chimeric controls were tested for their infectivity on U373-X4 and -R5 reporter cells. Limiting serial dilutions of either PBMC culture supernatants or infected PBMC stocks were used to determine their infectivity. Viral stock concentrations were as follows for cell-free virus (as expressed in ng/ml of p24) and for PBMC infected with chimeric viruses (as expressed in number of proviral DNA copies for 5×10^5 total cells), respectively: 1.15 and 5.9×10^4 for strain 594, 1.10 and 2.7×10^4 for strain 705, 1.52 and 1.6×10^4 for strain 728, and 1.34 and $3{,}3\times10^4$ for strain 804.

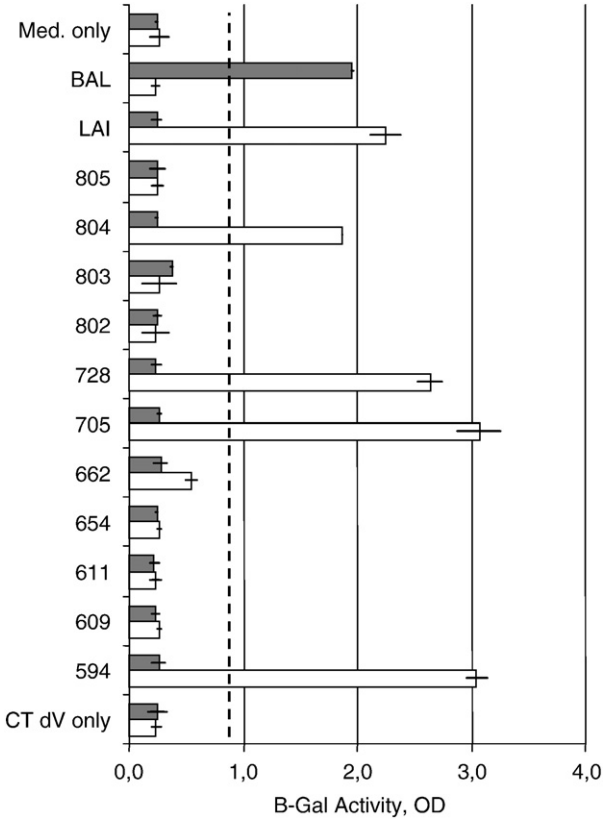

Fig. 1. Determination of viral co-receptor usage of chimeric viruses from 11 seminal plasma samples not consecutively numbered 594 to 805 and successfully amplified by nested PCR performed in the V1–V3 region. The experiments were performed after 16 days of amplification by co-culture with PBMCs as measured by induction of β-Gal activity 48 h after virus–cell incubation for parallel cultures of U373-X4 (open bars) and U373-R5 (grey bars) reporter cells. 'Vector only' and 'Medium only' refer to control conditions in which cells were incubated with supernatants from 293T cells transfected with the pNL 43 ΔV vector only and with cell culture medium only, respectively. Optical densities greater than twice the background values were considered positive (dotted lines). Results shown are the mean of three replicates per measure.

Tropism characterization

To assess the absence of divergence during transfection and amplification steps, the sequences of the V3 loop of the replicative chimeric viruses (4 seminal strains and controls) were compared with those from the corresponding initial V1–V3 RT-PCR products. No differences were encountered for any strain (data not shown). In order to compare the observed phenotropism of the chimeric strains with their genotype profile, sequencing of the V1–V3 region of the *env* gene was performed on PCR products amplified from infected PBMC (Table 1) and the genotypic tropism was predicted using a defined algorithm (http://www.geno2pheno.org/cgi-bin/geno2pheno.pl). Globally, a consensus was observed between this analysis and the TRT. However, two seminal clones were found to exhibit discordant profiles: R5 from V3 sequence-based prediction and X4 from the TRT for strain 594 and the opposite pattern for sub-clone 654–5.

As shown in panels A and E of Fig. 2, the presence of viral p24 antigen was detectable at the surface of U373-X4 cells incubated with chimeric X4-tropic strains LAI or seminal strain 728 by confocal

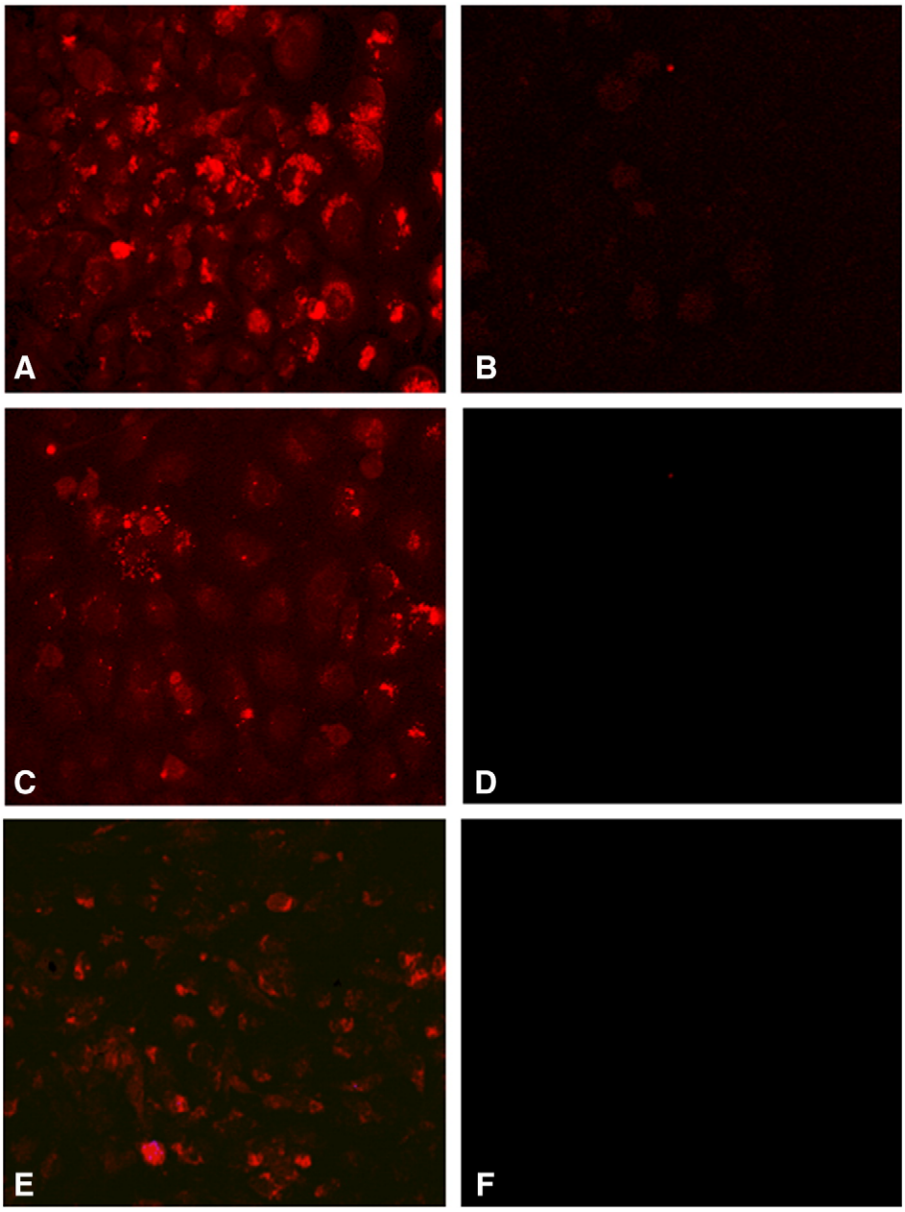

Fig. 2. Visualization of viral infection of U373 cells by confocal microscopy. The presence of p24 antigen (shown in red) was detectable at the surface of the cells after 1 h of incubation of LAI ΔV with the U373-X4 cell line (panel A), BAL ΔV with the U373-R5 cell line (panel C) or the chimeric strain 728 with the U373-X4 cell line (panel E). No signal was seen if viruses were incubated with the U373 cell line expressing the non-appropriate co-receptor, as LAI ΔV with the U373-R5 cell line (panel B). The p24 signal was lost if virus-containing supernatants (BAL ΔV in panel D and strain 728 in panel F) were pre-incubated for 30 min with suramin.

microscopy. Similar images were obtained for R5-tropic strains incubated with U373-R5 cells (Fig. 2C). This signal was lost if the virus-containing supernatants were pre-incubated with suramin (Figs. 2D and F), suggesting the presence of a functional V3 loop region for the recombinant viruses and its involvement in virus–cell interactions. A lack of p24 signal was also observed if U373 cells expressing an inappropriate co-receptor were incubated with these same viruses (Fig. 2B), indicating that these interactions were co-receptor-specific.

Discussion

Diverse tissues of the genital tract can serve as sites of HIV-1 replication and these sites are likely to differ from the peripheral environment in terms of target cell characteristics and immunological pressures (Coombs et al., 2001; Kiessling, 1992; Paranjpe et al., 2002). These differences have been shown further by studies dealing with the low correlation in some patients between viral loads in semen and blood, both in terms of RNA and provirus (Ball et al., 1999; Bourlet et al., 2001; Tachet et al., 1999). Virus replicating within the genital tract can therefore develop distinct genetic characteristics (Byrn et al., 1997; Delwart et al., 1998; Ghosn et al., 2004b; Ping et al., 2000 and this study). Moreover, genotypic differences in the C2/V5 region of gp120 of viruses from seminal cells and plasma from the same patient (Paranjpe et al., 2002) suggest the existence of a sub-compartmentalisation of HIV within mucosal secretions of the genital tract; the round cells and seminal plasma can be considered as two separate compartments, each containing distinct viral variants. In addition, efficiencies of drug penetration may also differ and although antiretroviral therapy appears to influence HIV levels in rectal and seminal fluids as well as in blood, higher RNA levels have been observed in mucosal secretions, highlighting the importance of anatomic viral compartmentalisation in the selection of mucosal-specific resistance patterns (Ghosn et al., 2004a, b).

Given that there is evidence of genetic differences between blood and semen-derived HIV-1 strains, it would seem pertinent to use viruses of seminal origin in *in vitro* or *ex vivo* studies dealing with interactions between vaginal mucosa and virus. Unfortunately, the amplification of HIV-1 strains from seminal samples by culture with PBMCs is fastidious and has low sensitivity, ranging from 9% to 55% (Coombs et al., 1998; Vernazza et al., 1997). In addition, extensive culturing of HIV-1 particles with PBMCs could lead to the selection of restricted strains that may also lead to the misrepresentation of the initial virus population. This is in part due to culturing PBMCs in the presence of interleukin-2 and phytohemagglutinin that may favour the expression of CXCR4 with respect to CCR5 (Bleul et al., 1997; Boyd et al., 1993).

In order to overcome the difficulties inherent in the isolation of seminal strains, we present here the adaptation of a technique originally developed to test the tropism of viral strains to construct replicative chimeric viruses expressing wild-type seminal HIV-1 envelope protein (Trouplin et al., 2001). From the eleven samples tested, the use of bulk PCR products allowed the obtention of four replicating strains (594, 705, 728, and 804). All of these four viruses were X4-tropic by the TRT, raising the question of a preferential selection of X4 variants in this protocol combining transfection with pNL43 and PBMC culture. However, sequencing results show that the V1–V3 regions of these X4-tropic chimeric strains were those that were present in the original bulk PCR products obtained from the semen of the five patients. In addition, these four samples were obtained from patients exhibiting a symptomatic phase of HIV infection (Table 1), making the detection of X4 strains plausible in this context. Even if it was necessary to cultivate the chimeric viruses with PBMC in order to increase the relatively low-level virus production observed with this transfection system, no genetic divergence was shown to be introduced by a single cycle of PBMC amplification, as assessed by sequencing of the V1–V3 region before and after culture.

Of the remaining six samples, two were included successfully in further sub-cloning experiments; one sample (611) gave four distinct R5-like V3 profiles and the other (654) two different profiles (one X4, one R5). These viruses were all revealed to be R5-tropic by the TRT but were capable of only one cycle of PBMC infection despite the absence of apparent anomalies (stop codons, frame-shifting deletions or insertions) in the proviral V1–V3 regions amplified from infected PBMCs. For these strains, a misalignment during the recombination of PCR products and deleted vector cannot be excluded. However, these viruses still represent useful strains for studying the initial steps of viral infection. Interestingly, a CRF02_AG strain was found able to produce chimeric viruses suggesting that this cloning strategy is not clade-dependant.

The chimeric viruses that were unable to infect target cells (662, 802, 803, and 805), even after sub-cloning, may represent defective circulating viruses present in the initial seminal samples that were selected by PCR and cloning steps. It has been shown that V3 mutations can lead to a major loss of entry fitness or even to lethality or that V1/V2 mutations in the absence of V3 mutations can alter co-receptor usage (Pastore et al., 2006). Also, it cannot be excluded that some primary sequences may not be functional in the context of the NL43 vector used here.

As already mentioned, some discrepancies were observed in this study between the actual viral phenotropism determined by the TRT and the genotropism predicted from the amino acid composition of the corresponding V3 loop (for a recent overview see (Sander et al., 2007; Xu et al., 2007)), confirming that other regions of gp160 are implicated in co-receptor usage, as recently described (Huang et al., 2008; Taylor et al., 2008). Given that mathematical-based prediction algorithms are not 100% reliable, greater weight must obviously be given to actual phenotypic results for the characterisation of these chimeric strains. At a therapeutic level, this observation may have important consequences for determining which patients may benefit from the use of entry inhibitors able to interact with a single class of co-receptor (Van Baelen et al., 2007).

In the absence of easily available cell-free or cell-associated primary isolates of seminal origin, most studies have turned to pure laboratory or blood-derived strains that may be weakly representative of the actual infectious, circulating strains responsible for the majority of HIV infections worldwide. The biological determinants that influence the heterosexual transmission of different viral strains from the seminal tract are still poorly understood. To our knowledge, previous studies dealing with interactions between mucosa and virus have never used primary isolates from seminal origin but laboratory or blood-derived isolates (Berlier et al., 2005; Dezzutti et al., 2001; Fotopoulos et al., 2002; Gupta et al., 2002; Hocini and Bomsel, 1999; Meng et al., 2002; Van Herrewege et al., 2007; Wu et al., 2003). Therefore, it would appear crucial to use seminal strains for the study of heterosexual transmission in *in vitro* models, at least to confirm results obtained with commonly used laboratory strains. As it would seem that the transfer of virus from infected cells present in the semen is the most efficient form of viral transmission and therefore route of primary infection (Tan and Phillips, 1996), it is also necessary to test any *in vitro* model system using infected cells. These replicative chimeric seminal viruses represent useful tools for the study of the heterosexual transmission of HIV-1 using models of genital barriers, testing of microbicide activity and for mucosal immunity.

Materials and methods

Seminal samples and HIV-1 strains

Seminal samples were obtained from 11 HIV-1 infected males followed at the Department of Infectious Diseases of the University Hospital of Saint-Etienne and tested positive by RT-PCR. As shown in Table 1, these subjects presented a blood viral load of more than 1000 copies/ml. All of them gave a written, informed consent. The cell-free fraction of seminal plasma was separated from whole semen by centrifugation for 30 min at 800 $\times g$ and stored at −80 °C until use. Two laboratory strains, HIV-1_{LAI} (X4-lymphocytotropic) and HIV-1_{BAL} (R5-monocytotropic) were used as controls.

Cell culture

The 293-T cell line was cultured in Eagle's minimum essential medium supplemented with 10% foetal calf serum (FCS) and antibiotics (Sigma Aldrich, Saint-Quentin Fallavier, France). U373MG-CD4 cells, a kind gift from M. Alizon (INSERM U332, Paris, France), are transfected with an expression vector for one of the chemokine receptors CCR5 or CXCR4 and contain an HIV-1 long terminal repeat (LTR)-LacZ cassette allowing the detection of infection via the Tat-induced expression of β-galactosidase (β-Gal). Referred to here as U373-R5 and U373-X4 cells, respectively, they were cultured in Dulbecco modified Eagle medium supplemented with 10% FCS and antibiotics in the presence of 10 μg/ml of puromycine and 100 μg/ml of hygromycin-B (Labrosse et al., 1998). Human PBMCs, obtained after Ficoll separation from leukocyte concentrates from healthy donors, were routinely cultured in RPMI 1640 medium supplemented with 10% FCS and antibiotics; their activation was carried out 2 days before virus infection by the addition of 5 μg/ml of PHA-P (Sigma-Aldrich) and 20 μg/ml of IL-2 (AbCys, Paris, France).

Amplification of the V1–V3 region of the HIV-1 envelope

Total RNA was purified from seminal plasma samples, blood plasma or from culture supernatants using the QIAamp Viral RNA mini kit (Qiagen, Courtaboeuf, France). An initial reverse transcription-PCR was carried out, followed by a nested PCR, according to the protocol of Trouplin et al. (2001). Both PCRs were performed using the Expand High Fidelity PCR system (Roche Diagnostics, Meylan, France). By this approach, 940-bp-long products that span the V1–V3 region deleted

from the 43 ΔV vector were obtained, with approximately 150-bp extensions on each side to allow homologous recombination during transfection. PCR products were verified for their size by agarose gel electrophoresis and were purified by the Qiagen PCR purification kit prior to use in transfection experiments.

Production of recombinant viruses by transfection

Recombinant viruses exhibiting the V1–V3 region of the HIV-1 *env* gene from seminal strains were produced by co-transfection in 293T cells of PCR products with the HIV-1 genomic construct V1–V3 deleted vector (43 ΔV), as described previously (Trouplin et al., 2001). The 43 ΔV vector was a kind gift from F. Mammano (Pasteur Institute, Paris, France). As control, cells were transfected with the 43 ΔV vector in the absence of PCR product. Five ml of virus-containing supernatant was collected 48–72 h after transfection, clarified by centrifugation and tested by p24 ELISA assay.

Quantification of p24 antigen by ELISA

The following monoclonal antibodies directed against the p24 protein of HIV-1 were used in a classical sandwich ELISA on 96-well microplates; D7320 and BC 1071 (Aalto Bio Reagents, Dublin, Ireland). Serial dilutions of a recombinant p24 protein (5109, Aalto Bio Reagents) were used to establish a concentration gradient for quantitative analysis. An ELISA Amplification System® (Invitrogen, Cergy-Pontoise, France) was used to enhance assay performance following manufacturer's instructions. The sensitivity of this assay was estimated to be approximately 40 pg/ml of p24 protein.

Chimeric virus amplification and titration

Virus-containing supernatant from co-transfected 293-T cells was used to infect PHA/IL-2 stimulated cultures of PBMCs. To assess viral production, a sample of culture supernatant was taken every day for 16 days, cleared by centrifugation and quantified by p24 ELISA. PBMC supernatants presenting the highest p24 load (expressed in ng/ml) were further controlled for RNA viral load (expressed in copies/ml) by using a real-time PCR assay (HIV RealTime, m2000rt, Abbott Molecular, Rungis, France). The amount of proviral DNA in the corresponding PBMC fractions collected after 48 h of infection was determined as described by O'Doherty et al. (2002).

Determination of viral phenotropism by the TRT

The determination of the replicative capacity of the chimeric strains was also assessed using the TRT as detailed in Trouplin et al. (2001). Co-receptor usage was determined by measurement of CPRG in the two target cell lines by comparison with cells exposed to supernatant from cells transfected with the 43 ΔV vector in the absence of PCR products (background). Optical densities greater than twice the background value were considered positive. Results were recorded from at least three independent experiments.

Sequencing of the V1–V3 region

Sequencing of the V1–V3 region of the *env* gene was performed on the initial V1–V3 RT-PCR products and, as a control of viral sequence divergence, on V1–V3 PCR products amplified from infected PBMCs. Following purification of PCR products (QIAquick gel extraction kit, Qiagen), a 900 bp-long fragment of the V1–V3 region was sequenced using the primers E20 and E115 (Trouplin et al., 2001) and the DTCS kit (Beckman-Coulter, Villepinte, France) on a Beckman 2000 automated sequencer following manufacturer's recommendations. The sequences were analysed by performing multiple sequence alignment using CINEMA 5 software (version 0.2.1. BETA, http://aig.cs.man.ac.uk/utopia) with minor adjustments. Vector clone pNL43 and HIV-1_{BAL} were used as references. All V1–V3 sequences from seminal strains reported here have been submitted to the GenBank database with the following accession numbers: EU977327 to EU977341, consecutively.

Subcloning of the V1–V3 region

RT-PCR-amplified material from six seminal plasma samples was sub-cloned using the Qiagen PCR Cloning kit according to the manufacturer's instructions. To increase the likelihood of amplifying and detecting different viral variants present in seminal plasma samples, a mixture of at least 6 nested PCR products was used for cloning. Verification of the clones was carried out by PCR using the primers E20 and E115 on single colonies grown overnight in Luria Bertani medium following purification with the QIAprep Miniprep kit (Qiagen) according to manufacturer's instructions.

Confocal microscopy

Cells were grown to 70% confluence on glass coverslips in 24-well plates before incubation at 37 °C with viral strains for the indicated time. Cells were fixed in 3.7% paraformaldehyde-PBS for 12 h and then incubated for 20 min in 50 mM NH_4Cl to quench free aldehydes. After an incubation of 20 min in 0.4% Triton X-100-PBS, the cells were incubated for 1 h with relevant antibodies (HIV-1 p24 Monoclonal Antibody 183-H12-5C, AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH; coupled with Alexa Fluor 555, Invitrogen, and for nuclear staining 7-AAD, Becton Dickinson, Le Pont-De-Claix, France) in PBS with 10% FCS. Following washing steps, coverslips were mounted using Fluoprep mounting medium (Biomerieux, Marcy-l'Etoile, France) and left overnight at 4 °C. Cells were analysed with a LEICA TCS-SP2 confocal scanning laser microscope used with pulsed near infrared excitation (multiphoton mode) and software. The excitation laser was a COHERENT MIRA 900 with a 76 MHz repetition rate and a 790 nm wavelength. Image stacks were further analysed using ImageJ software (Abramoff et al., 2004). In certain experiments, virus containing supernatants were pre-incubated for 30 min with 5 mg/ml of suramin (Sigma-Aldrich), a polyanionic compound known to interfere with the V3 loop of gp120 (Yahi et al., 1994).

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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