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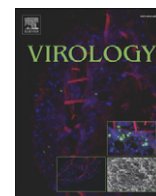
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Envelope glycoproteins of Human Immunodeficiency Virus type 1 variants issued from mother–infant pairs display a wide spectrum of biological properties

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ABSTRACT

Several studies have shown that the early virus population present in HIV-1 infected infants usually is homogeneous when compared to the highly diversified viral population present at delivery in their mothers. We explored the antigenic and functional properties of pseudotyped viruses expressing gp120 encoded by *env* clones issued from four mother–infant pairs infected by CRF01_AE viruses. We compared their sensitivity to neutralization and to entry inhibitors, their infectivity levels and the Env processing and incorporation levels. We found that both transmitted viruses present in infants and the variants present in their chronically infected mothers display a wide spectrum of biological properties that could not distinguish between them. In contrast, we found that all the transmitted viruses in the infants were more sensitive to neutralization by PG9 and PG16 than the maternal variants, an observation that may have implications for the development of prophylactic strategies to prevent mother-to-child transmission.

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Introduction

Mother-to-child transmission (MTCT) is the leading source of human immunodeficiency virus (HIV) infection in children. In the absence of anti-retroviral prophylaxis, transmission can occur during pregnancy (*in utero*), during labor and delivery (intrapartum), or postnatally through breastfeeding (Scarlati, 2004). Although infants have been found occasionally to be infected by a heterogeneous population of multiple maternal viral variants, molecular studies of MTCT have shown that, despite a heterogeneous viral population in the mother, homogeneous viral variants are generally transmitted to the infant, suggesting the selection of a limited number of maternal viral variants for establishment of a new infection in the infant (Ahmad et al., 1995; Dickover et al., 2001; Kishko et al., 2011; Lamers et al., 1994; Pasquier et al., 1998; Russell et al., 2011; Samleerat et al., 2008; Scarlati et al., 1993b; Verhofstede et al., 2003; Wolinsky et al., 1992; Zhang et al., 2010b).

Maternal neutralizing antibodies (Nabs) are among the selective factors that are potentially responsible for this genetic bottleneck. Maternal antibodies of the IgG class cross the placenta into the fetal bloodstream, reaching high levels in the fetus at the end of pregnancy and protecting the infant against infection by numerous pathogens (Englund et al., 1998; Safrit et al., 2004). Therefore, MTCT of HIV-1 provides a model for studying the role of passively acquired antibodies present in the infant prior to virus exposure. However, reported studies have yielded conflicting results. Some studies have suggested a role of maternal Nabs in reducing MTCT, showing that non-transmitting mothers had more frequently detected or higher Nab responses than mothers who transmitted the virus to their infant (Barin et al., 2006; Bongertz et al., 2002; Lathey et al., 1999; Samleerat et al., 2009; Scarlati et al., 1993a), or that viruses transmitted to infants are escape variants resistant to autologous maternal serum (Dickover et al., 2006; Wu et al., 2006; Zhang et al., 2010a). In contrast, others did not observe any difference neither in breadth or potency of neutralizing antibodies between sera from transmitting and non-transmitting mothers (Guevara et al., 2002; Hengel et al., 1998; Husson et al., 1995; Russell et al., 2011), nor in the sensitivity to neutralization between transmitted infant variants and maternal variants (Kishko et al., 2011; Russell et al., 2011). In addition, a recent study exploring the role of passively acquired HIV antibodies in exposed infants during

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breastfeeding suggested that the breadth and potency of the heterologous antibody response does not predict protection (Lynch et al., 2011).

Very few studies have been done focusing on the viral characteristics associated with MTCT, others than neutralization sensitivity. A correlation between HIV-1 transmission to infants and replicative fitness of transmitted viruses was suggested (Kong et al., 2008) but not confirmed (Kishko et al., 2011). Independently, several studies performed mainly on HIV-1 strains of subtypes A and C suggested that variants with shorter variable loops lengths and fewer potential N-linked glycosylation sites (PNGS) encoded by their *env* gene were selected during MTCT (Russell et al., 2011; Wu et al., 2006; Zhang et al., 2010b). In contrast, we and others did not observe these characteristics among *env* genes from mother–infant pairs infected with variants of B and CRF01_AE clades (Kishko et al., 2011; Samleerat et al., 2008). These discordant results may suggest that, similarly to what was observed during horizontal transmission, molecular properties linked to transmissibility could be subtype-specific (Chohan et al., 2005; Derdeyn et al., 2004; Frost et al., 2005). Nevertheless, in our study performed on CRF01-AE *env* variants of mother–infant pairs, we found that two PNGS, N301 in V3 and N384 in C3, were conserved in almost all infants' variants but were not uniformly present in variants from mothers. We hypothesized that these two PNGS may confer a selective advantage for transmission of the virus to the infants (Samleerat et al., 2008).

In the present study, we compared the biological properties of the virus conferred by the envelope of maternal and infant viral variants issued from four mother–infant pairs infected by HIV-1 of the CRF01_AE clade, in order to explore their association with the restrictive transmission of the virus. A better understanding of antigenic and functional properties of transmitted viral variants may help to the development of vaccines or improved prophylactic strategies to prevent MTCT.

Results

HIV-1 mother–infant pairs (MIPs)

We selected HIV-1 CRF01_AE *env* sequences (V1 to V5 region of gp120) from four previously described MIPs [0377, 0858, 0978 and 1021; (Samleerat et al., 2008)]. Maternal *env* sequences were obtained from peripheral blood samples collected at delivery and infant *env* sequences from plasma samples obtained at the first time point at which the HIV-1 DNA PCR results was positive. One infant (0858) was positive at birth for HIV-1 DNA, indicating that he was infected *in utero* (Table 1). The three remaining infants (0377, 0978 and 1021) were HIV-1 DNA negative at birth but were found positive at 71, 55 and 67 days after birth, respectively. Because of these 3 infants were not breastfed, HIV-1 transmission occurred during delivery (intrapartum). Thirty-seven clones (9 from MIP 0377, 11 from MIP 0858, 12 from MIP 0978 and 5 from MIP 1021) were selected based on the fact that they were representative of the diversity of the variants

present in the mothers and their babies, and that they possessed or not the PNGS at positions N301 and N384 (Fig. 1). Chimeric *env* genes were constructed by insertion of the V1 to V5 *env* fragment in a NL4.3 *env* backbone as previously described (Braibant et al., 2010), and the 37 corresponding Env-pseudotyped viruses were generated. Seventeen of them were infectious in TZM-bl cells: 6 from MIP 0377, 2 from MIP 0858, 6 from MIP 0978 and 3 from MIP 1021 (Table 2, Fig. 1).

Sensitivity to neutralization by maternal plasma

We determined the sensitivity to neutralization of mother- and infant-derived Env-pseudotyped viruses with the maternal plasma collected during pregnancy just before the initiation of zidovudine (ZDV) prophylaxis, 4 to 11 weeks before delivery. Maternal variants issued from MIPs 0377, 0978 and 1021 were relatively resistant to autologous neutralization. Indeed, 0377 maternal clones presented a low sensitivity to neutralization (IC₅₀ range: <20–103.1) and 0978 and 1021 maternal clones were particularly resistant to autologous antibodies, failing to reach 50% neutralization even using a 1:20 dilution of plasma, the highest plasma concentration tested (Table 2). Infant variants were also generally resistant to maternal plasma (IC₅₀ range: <20–28.7), except a single clone from pair 0978, clone 0978-I2, which on contrary, displayed a high sensitivity to maternal plasma (IC₅₀: 883; Table 2). In contrast, the two maternal clones from MIP 0858, presented a high neutralization-sensitivity to maternal autologous plasma with IC₅₀ values of 1197 and 1642. However, due to the lack of infectious infant clones for this pair, we were not able to compare their susceptibility to the transmitted variant(s). When the four pairs were considered together, we did not observe any difference in sensitivity to autologous plasmas between mother and infant variants ($P=0.38$, mixed model test; Fig. 2A).

Sensitivity to neutralization by heterologous sera

We investigated the sensitivity of maternal and infant variants to neutralization by a pool of ten heterologous broadly neutralizing sera selected from patients infected by CRF01_AE viruses in a previous study (Samleerat et al., 2009). The seventeen clones presented a broad and continuous range of sensitivity to heterologous antibodies (IC₅₀ range: 123–5543; Table 2). Among MIP 0377, the infant clone presented a higher neutralization-sensitivity (IC₅₀: 1056) compared to the corresponding maternal clones (IC₅₀ range: 123–339). On the contrary, maternal clones from pairs 0978 and 1021 presented similar sensitivity to heterologous neutralization (0978 IC₅₀ range: 626–5543, 1021 IC₅₀: 990) compared to infants clones (0978 IC₅₀ range: 2277–4150; 1021 IC₅₀: 585 and 746). When the four pairs were considered together, we did not observe any difference in sensitivity to heterologous plasmas between mother and infant variants ($P=0.97$, mixed model test; Fig. 2B).

Sensitivity to neutralization by monoclonal antibodies

We tested the sensitivity of our pseudotyped viruses to neutralization by the broadly neutralizing human monoclonal antibodies (mAbs) b12, PG9 and PG16. b12 is directed against an epitope overlapping the CD4-binding site (CD4BS) (Burton et al., 1994; Saphire et al., 2001), whereas PG9 and PG16 recognize a quaternary neutralizing epitope formed from conserved regions of V1/V2 and V3 variable loops (Pancera et al., 2010; Walker et al., 2009). All maternal and infant clones from MIPs 0377, 0978 and 1021, displayed a high level of resistance to neutralization by mAb b12 (IC₅₀>50 µg/mL), whereas the two maternal clones from pair 0858 were highly sensitive to b12 (IC₅₀<0.1 µg/mL; Table 2). On the contrary, we observed more heterogeneous results for PG9 and PG16 neutralization. Maternal clones issued from MIPs 0377, 0978 and 1021 displayed a broad and continuous range of sensitivity to both PG9 (IC₅₀ range: 0.07 to

Table 1
Characteristics of mother–infant pairs.

MIP	Subject	First positive	Transmission	Number clones selected	Number clones infectious
0377	Mother	–	ip	8	5
	Infant	71 days		1	1
0978	Mother	–	ip	7	3
	Infant	55 days		5	3
1021	Mother	–	ip	2	1
	Infant	67 days		3	2
0858	Mother	–	iu	6	2
	Infant	at birth		5	0

ip: intrapartum.

iu: *in utero*.

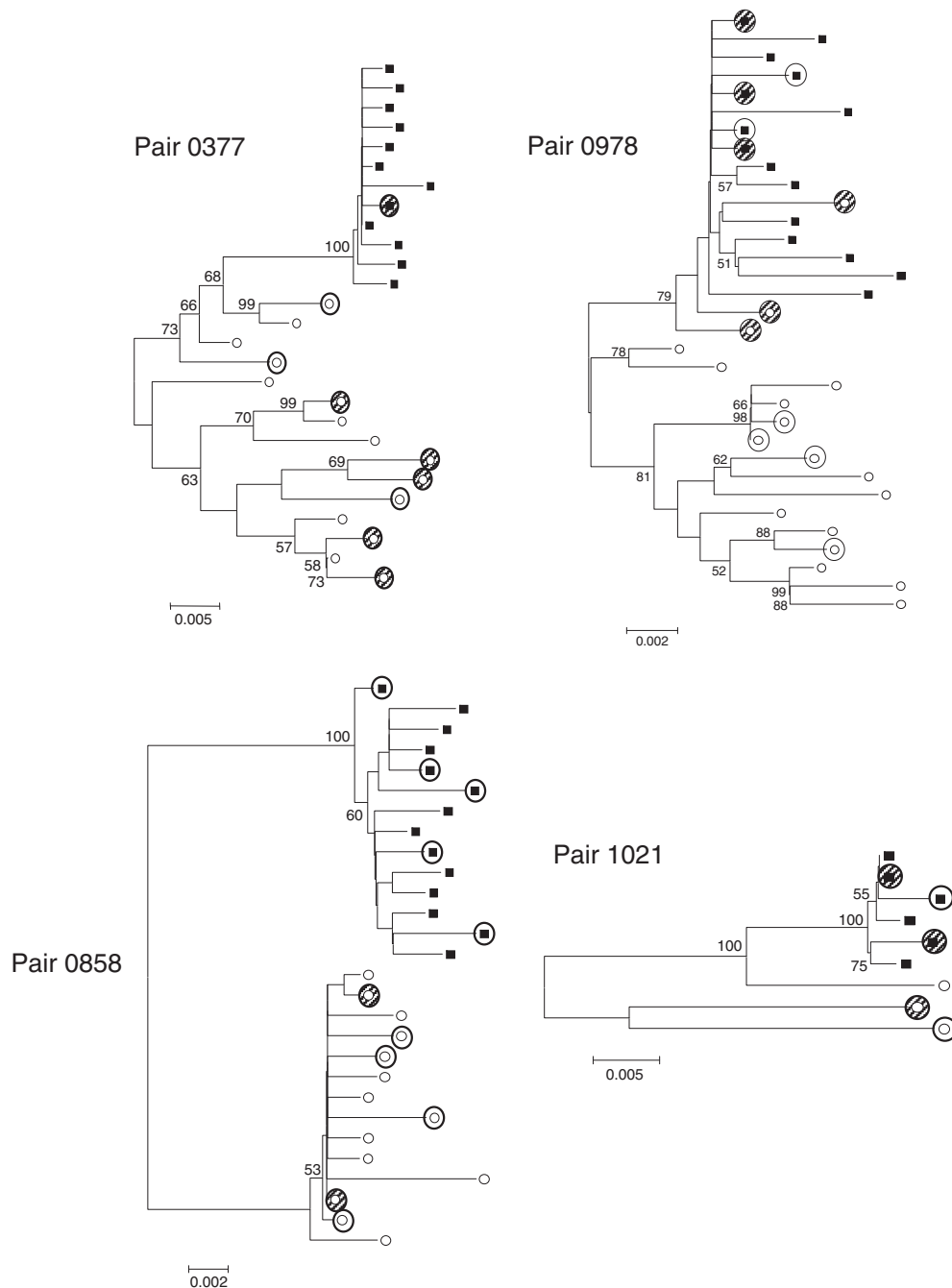


Fig. 1. Phylogenetic analysis of *env* sequences derived from mother–infant pairs 0377, 0858, 0978 and 1021. A distance scale is given for each neighbor-joining tree. Bootstrap values are expressed as percentages per 1000 replicates, and values above 50% are indicated on nodes. Each symbol denotes a single *env* sequence: ○, maternal sequence; ■, infant sequence. The 37 clones selected for generating Env-pseudotyped viruses are encircled. Among these, the 17 infectious clones are hatched.

>10 µg/mL) and PG16 (IC₅₀ range: 0.03 to >10 µg/mL) whereas all infant clones were highly sensitive to both mAbs (PG9 IC₅₀ range: 0.03–0.18 µg/mL; PG16 IC₅₀ range: 0.01–0.36 µg/mL). When the four MIPs were considered in aggregate, infant clones were significantly more sensitive to PG9 ($P=0.04$, mixed model test; Fig. 2C) and PG16 ($P<0.01$, mixed model test; Fig. 2D) compared to maternal clones.

Viral infectivity in TZM-bl cells and PBMC

A selective advantage for the variants transmitted to the infants could be the consequence of a higher viral infectivity. Therefore, we

investigated the capacity of pseudotyped viruses to infect TZM-bl indicator cells and primary stimulated-peripheral blood mononuclear cells (PBMCs) in a single round of infection. Infectivity level of each pseudotyped virus, whose quantity was normalized for p24 amount, was compared with that of a pseudotyped virus expressing the envelope of the NL4-3 reference strain (Table 2). Although infectivity levels were globally higher in TZM-bl cells than in PBMCs, a high correlation was observed between the two cell types ($r=0.93$; $P<0.0001$; Fig. 3C). A considerable variability in infectivity levels was observed among maternal clones from MIP 0377 with two clones, 0377-M2/M5, being poorly infectious and three clones, 0377-M1/M3/M4, being highly infectious (Table 2). Similarly, a high

Table 2

Summary of the biological properties of mother- and infant-derived Env-pseudotyped viruses.

MIP	env clone (Genbank ID)	N301	N384	Sensitivity to neutralization by					Functional properties				
				Autologous plasma	Heterologous plasma	b12 (μg/ml)	PG9 (μg/ml)	PG16 (μg/ml)	Tropism	sCD4 (μg/ml)	TAK-779 (μg/ml)	TZM-bl infectivity	PBMC infectivity
377	0377-M1 (EU031223)	–	+	103.1	212	> 50	> 10	> 10	R5	1.74	0.039	16.8	0.67
	0377-M2 (HQ875343)	+	–	31.9	339	> 50	0.31	1.98	R5	4.15	0.038	0.6	0.09
	0377-M3 (EU031226)	+	–	20.4	123	> 50	1.46	3.31	R5	> 10	0.036	43.7	4.13
	0377-M4 (EU0311227)	+	–	22.3	148	> 50	2.43	7.58	R5	> 10	0.056	31.3	3.27
	0377-M5 (HQ875344)	+	–	< 20	214	> 50	> 10	> 10	R5	3.19	0.038	0.3	0.05
	0377-I1 (JQ003579)	+	+	28.7	1056	> 50	0.03	0.01	R5	2.97	0.019	4.5	0.25
978	0978-M1 (HQ875346)	+	+	< 20	626	> 50	0.07	0.03	R5	5.71	0.086	3.1	0.22
	0978-M2 (EU031126)	+	+	< 20	5543	> 50	0.13	0.07	R5	> 10	0.078	2.1	0.11
	0978-M3 (HQ875347)	+	+	< 20	3900	> 50	> 10	> 10	R5	0.15	0.021	0.4	0.10
	0978-I1 (EU031128)	+	+	< 20	2489	> 50	0.10	0.06	R5	> 10	0.062	5.7	0.43
	0978-I2 (EU031141)	+	+	883	2277	> 50	0.18	0.16	R5	0.23	0.100	1.6	0.16
	0978-I3 (EU031130)	+	+	< 20	4150	> 50	0.11	0.04	R5	> 10	0.044	3.1	0.19
1021	1021-M1 (JQ003580)	+	+	< 20	990	> 50	0.86	0.48	R5	> 10	0.027	43.9	0.8
	1021-I1 (EU031153)	+	+	< 20	585	> 50	0.18	0.34	R5	3.59	0.039	45.1	0.64
	1021-I2 (EU031150)	+	+	< 20	746	> 50	0.14	0.36	R5	2.36	0.048	1.9	0.11
858	0858-M1 (EU031374)	–	–	1197	2709	< 0.1	0.29	> 10	R5	> 10	0.008	4.6	0.19
	0858-M2 (HQ875345)	–	–	1642	2112	< 0.1	> 10	> 10	R5	0.35	0.005	1.1	0.15

The mAb neutralization and sCD4 inhibitory titers are color coded as follows: a red box indicates an $IC_{50} \leq 0.1 \mu\text{g/mL}$, an orange box indicates $0.1 \mu\text{g/mL} < IC_{50} \leq 10 \mu\text{g/mL}$ and a yellow box indicates an $IC_{50} > 10 \mu\text{g/mL}$. For the plasma, the color codes are as follows: a red box indicates an $IC_{50} \geq 1:100$ dilution, an orange box indicates $1:20 \leq IC_{50} < 1:100$ and a yellow box indicates an $IC_{50} < 1:20$. For the TZM-bl and PBMC infectivity, the color codes are as follows: a dark green box indicates an infectivity ratio (defined as mean RLU values obtained for the pseudotyped virus compared to mean RLU values obtained with the NL4-3 reference virus) ≥ 10 , a medium green box indicates $1.0 \leq \text{ratio} < 10$ and a light green box indicates a ratio < 1 . Infant clones are highlighted in gray.

variability was observed in infant clones from MIP 1021 with clone 1021-I1 being highly infectious when compared to clone 1021-I2, much less infectious (Table 2). The infectivity levels of clones from MIP 0978 were more homogeneous for both maternal and infant

clones (Table 2). When the four MIPs were considered together, we did not observe any difference in infectivity between maternal and infant clones in TZM-bl cells ($P = 0.60$, mixed model test; Fig. 3A), nor in PBMC cells ($P = 0.31$, mixed model test; Fig. 3B).

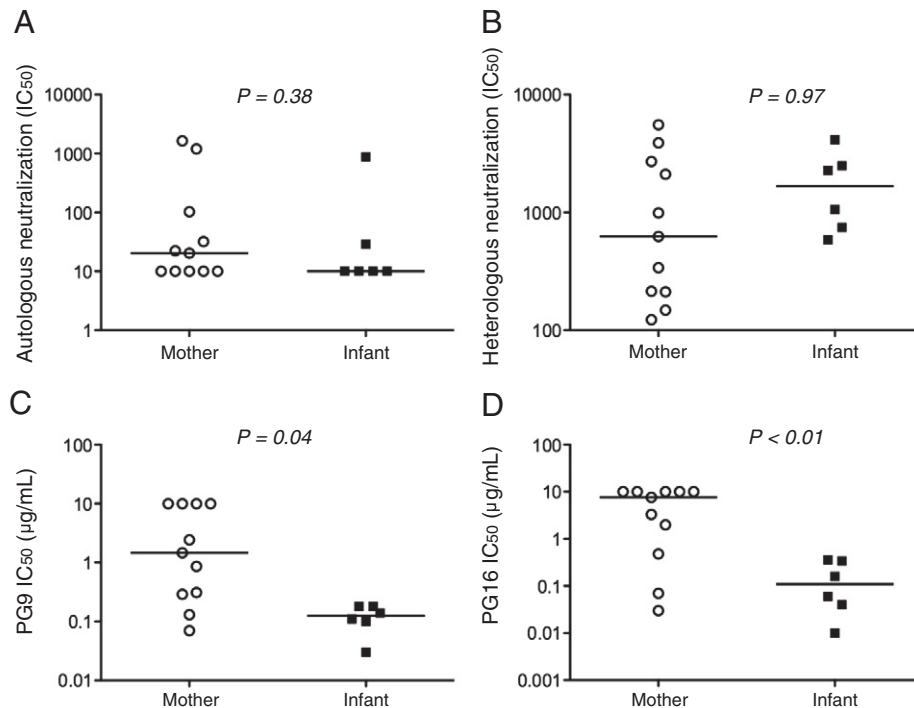


Fig. 2. Sensitivity to neutralization of mother- and infant-derived Env-pseudotyped viruses. The neutralization sensitivity of maternal- (○) and infant- (■) derived Env-pseudotyped viruses to (A) autologous plasmas, (B) heterologous plasmas, (C) PG9 and (D) PG16 was determined using a luciferase reporter gene assay in TZM-bl cells. IC₅₀ values were defined as the reciprocal of the serum dilution or antibody concentration (μg/mL) that causes 50% inhibition of virus infection. The horizontal bars indicate the median IC₅₀ values. Comparisons between maternal and infant-derived Env-pseudotyped viruses were done using a mixed model test.

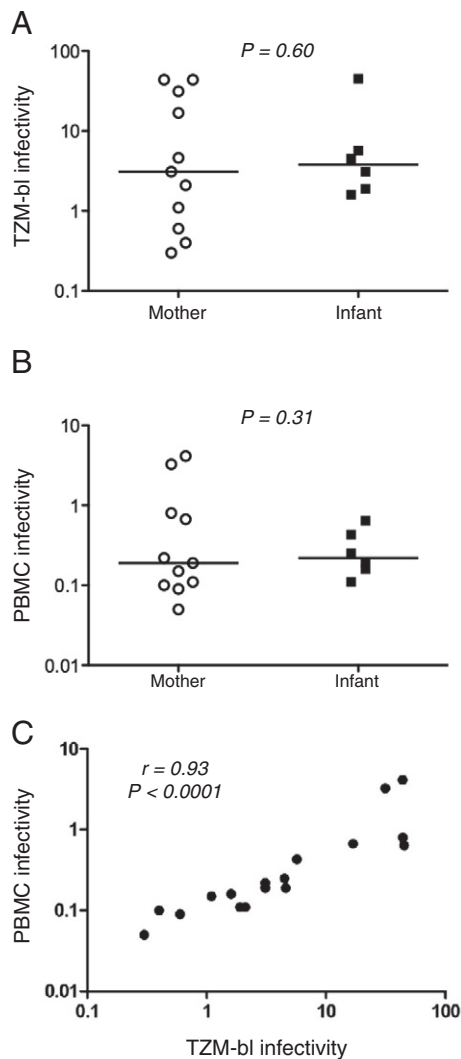


Fig. 3. Viral infectivity of mother- and infant-derived Env-pseudotyped viruses. The capacity of maternal- (○) and infant- (■) derived Env-pseudotyped viruses to infect (A) TZM-bl indicator cells and (B) primary stimulated-peripheral blood mononuclear cells (PBMCs) was evaluated in a single round of infection. Results are expressed as the ratio of mean RLU values obtained for each pseudotyped virus compared to mean RLU values obtained with the NL4.3 reference virus. Comparisons between maternal and infant-derived Env-pseudotyped viruses were done using a mixed model test. (C) Correlation between TZM-bl and PBMC infectivity. The correlation coefficient r and P values were generated using Spearman's correlation test.

Envelope processing and incorporation

To investigate whether the levels of Env processing and/or incorporation in virions contributed to differences in infectivity or had specific features in transmitted variants, we analyzed the expression of Env glycoproteins on maternal and infant pseudotyped viruses by western blot after electrophoresis under reducing and native conditions (Figs. 4A–B). Before electrophoresis, viral proteins from pelleted virions were solubilized and their quantity was normalized for p24 antigen (100 ng per well). Under reducing conditions and using polyclonal anti-gp120 antibody, we detected the expression of both mature gp120 and uncleaved gp160 precursor in almost clones (Fig. 4A). Despite some differences in the extent of processing between clones, we could not detect obvious pattern in Env processing that could distinguish infant from mother clones. Electrophoresis was performed under native conditions to quantify the incorporation of Env under its trimeric form in each variant. To avoid bias due to possible antigenic differences between the various envelopes, we used the anti-gp41

2 F5 and 4E10 monoclonal antibodies to detect the envelope spikes incorporated since all envelope glycoproteins were chimeras containing the same gp41 sequence derived from the NL4-3 virus. Using these antibodies, we detected gp120-gp41 trimers but also various forms of envelope glycoproteins, including gp120-gp41 monomers (~160 kDa) and gp41 trimers (~140 kDa) (Fig. 4B). Analysis by densitometry of the western blots showed that different levels of gp120-gp41 trimers were incorporated into viruses (Fig. 4B). For most viruses, the level of Env trimers incorporation correlated with the infectivity level, when analyzing the ratio trimer/p24 ($r = 0.82$, $P < 0.0001$; Fig. 4C). However, despite variability in incorporation levels of Env trimers into virions, these levels did not distinguish the maternal from the infant viruses ($P = 0.23$, mixed model test; Fig. 4D).

Sensitivity to entry inhibitors

The co-receptor usage of the infectious Env-pseudotyped viruses was evaluated using U373-MAGI cell lines that stably express the CD4 receptor and either the CCR5 or CXCR4 co-receptors. All viruses exhibited a CCR5 tropism. We next investigated the sensitivity of maternal and infant clones to soluble CD4 (sCD4) and TAK-779, a CCR5 antagonist. All maternal and infant exhibited a relatively homogeneous sensitivity to TAK-779, with IC_{50} ranging from 0.019 to 0.100 $\mu\text{g/mL}$. No statistically significant difference was observed between maternal and infant viruses ($P = 0.62$, mixed model test; Fig. 5A). Sensitivity to sCD4 was considerably heterogeneous within the four mother–infant pairs (IC_{50} range: 0.15 to $> 10 \mu\text{g/mL}$) (Table 2). When clones from the four MIPs were considered in aggregate, we did not observe any difference in sensitivity to sCD4 between maternal and infant clones ($P = 0.60$, mixed model test; Fig. 5B). The sensitivity to sCD4 was associated with infectivity: the most resistant variants were those that were most infectious ($r = 0.54$, $P = 0.02$; Fig. 5C). In contrast, the sensitivity to sCD4 was not associated with the level of Env trimers incorporation ($r = 0.25$, $P = 0.34$; Fig. 5D).

Antigenic and functional properties according to PNGS at positions N301 and N384

The two PNGS at positions 301 and 384 were present in all infant clones whereas they were not uniformly present in maternal clones. Maternal clones from pair 0377 harbored either N301 or N384, with one clone (0377-M1) $N301^- N384^+$ and four clones (0377-M2–M5) $N301^+ N384^-$. The two maternal clones from pair 0858 were $N301^- N384^-$ whereas the three maternal clones from pair 0978 and the unique maternal clone from pair 1021 were $N301^+ N384^+$. It should be noted that given the small number of functional pseudotyped viruses obtained, we could not test maternal clones from pairs 0978 and 1021 that were $N301^-$ and/or $N384^-$, nor infant clones from pair 0858 that were $N301^+ N384^+$. Nevertheless, our data suggested that the presence of N301 in presence or not of N384 conferred higher resistance to autologous neutralization ($P = 0.01$, Mann Whitney test). Indeed, maternal clones 0377-M2 to M5 ($N301^+ N384^-$) and infant clone 0377-I1 ($N301^+ N384^+$) presented a higher resistance to autologous neutralization (IC_{50} range: < 20 –31.9) compared to maternal clone 0377-M1 ($N301^- N384^+$; IC_{50} : 103.1). All maternal and infant clones from pairs 0978 and 1021 ($N301^+ N384^+$), except infant clone 0978-I2, were highly resistant to autologous maternal plasma ($IC_{50} < 20$) whereas maternal clones from pair 0858 ($N301^- N384^-$) were highly sensitive to autologous neutralization (IC_{50} : 1197 and 1642). Moreover, the absence of both PNGS at positions N301 and N384 compared to the absence of N301 alone, could increase neutralization sensitivity. Indeed, maternal clones from pair 0858 ($N301^- N384^-$) were more sensitive to autologous neutralization than maternal clones from pair 0377 ($N301^- N384^+$ or $N301^+ N384^-$). All together, these results may suggest that both N301 and N384 could be involved in sensitivity to autologous neutralization.

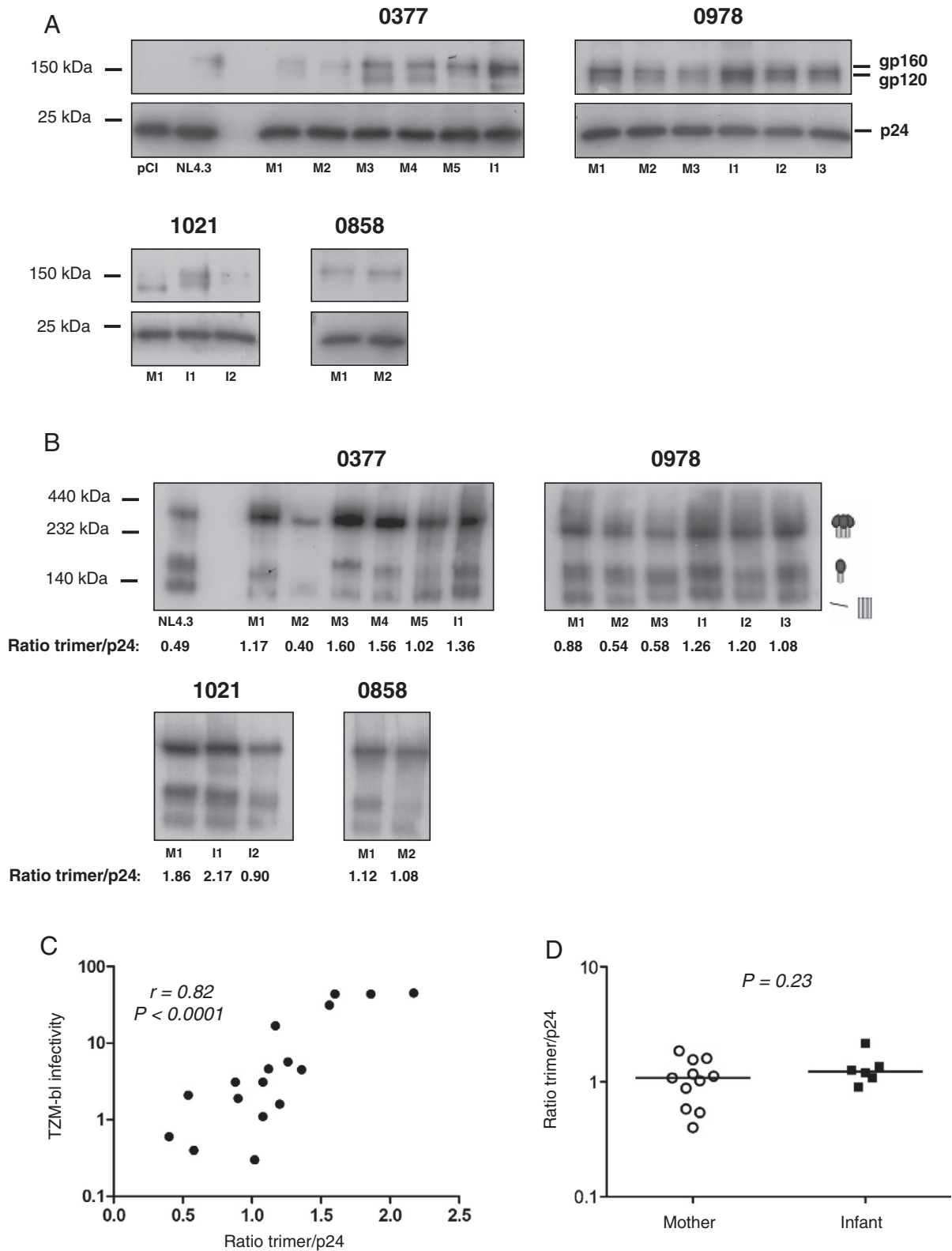


Fig. 4. Env processing and incorporation in mother- and infant-derived Env-pseudotyped viruses. Analysis of the Env glycoprotein composition was performed by western blotting after (A) SDS-PAGE under reducing conditions or (B) Blue-Native-PAGE (BN-PAGE) under native conditions. SDS-PAGE blots were revealed with polyclonal anti-gp120 or anti-p24 antibodies; BN-PAGE blots were revealed using anti-gp41 2F5 and 4E10 monoclonal antibodies (see [Materials and methods](#)). To quantify the incorporation of Env under its trimeric form, the gp160 trimer/p24 ratio was calculated for each Env-pseudotyped virus. (C) Correlation between trimers incorporation (gp160 trimer/p24 ratio) and viral infectivity in TZM-bl cells. The correlation coefficient r and P values were generated using Spearman's correlation test. (D) Comparison of trimers incorporation (gp160 trimer/p24 ratio) between maternal and infant-derived Env-pseudotyped viruses using a mixed model test. The horizontal bars indicate the median values of gp160 trimer/p24 ratios.

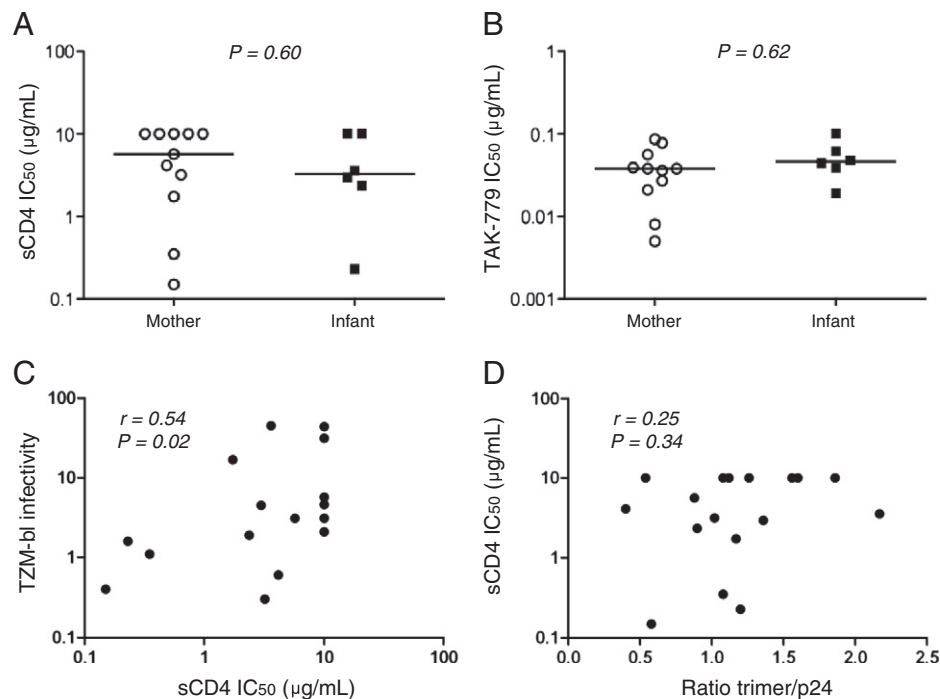


Fig. 5. Sensitivity to entry inhibitors of mother- and infant-derived Env-pseudotyped viruses. The sensitivity of maternal (○) and infant (■) clones to (A) sCD4 and (B) TAK-779 was determined using a luciferase reporter gene assay in T2M-bl cells. IC₅₀ values were defined as the inhibitor concentration (µg/mL) that causes 50% inhibition of virus infection. The horizontal bars indicate the median IC₅₀ values. Comparisons between maternal and infant-derived Env-pseudotyped viruses were done using a mixed model test. (C) Correlation between sensitivity to sCD4 and infectivity in T2M-bl cells. (D) Correlation between sensitivity of sCD4 and Env trimers density. The correlation coefficients r and P values were generated using Spearman's correlation test.

We next compared the functional properties according to the presence or not of PNGS at positions N301 and/or N384. We observed a wide range of sensitivity to sCD4 and infectivity levels whatever the presence or not of N301 (sCD4 IC₅₀ range: 0.15 to >10 µg/mL vs 0.35 to >10 µg/mL; T2M-bl cells range: 0.3 to 45.1 vs 1.1 to 16.8) or N384 (sCD4 IC₅₀ range: 0.15 to >10 µg/mL vs 0.35 to >10 µg/mL; T2M-bl ratio range: 0.4 to 45.1 vs 0.4 to 43.7). These results suggested that N301 and N384 PNGS did not seem to be involved in infectivity or sensitivity to sCD4.

Discussion

During perinatal transmission of HIV-1, acquisition of a homogeneous genetic restricted viral population has been regularly observed, suggesting the presence of selective pressures. The viral properties of perinatally transmitted viruses remain incompletely understood. In this study, we compared the biological properties of 17 Env-pseudotyped viruses derived from variants of mother–infant pairs infected by HIV-1 strains of the CRF01_AE clade. We determined their sensitivity to neutralization by autologous and heterologous sera as well as by broadly neutralizing mAbs. We did not find any significant difference in neutralization sensitivity between maternal and infant clones by either autologous or heterologous sera. However, it should be noted that in most cases maternal variants displayed a low or undetectable neutralizing sensitivity to autologous maternal plasma. As maternal plasma samples were collected before the timing at which *env* sequences were obtained, it may be possible that maternal clones had started to evolve to escape earlier antibodies. Nevertheless, one variant in an infant (clone 0978-I2 from pair 0978) was more sensitive to autologous maternal plasma than all tested maternal variants. Its presence in the infant suggested a low impact of maternal antibodies on the selection of transmitted variants. This observation confirmed recent studies suggesting that the genetic bottleneck in vertical transmission is not driven by selection of neutralization-resistant variants from the maternal viral population

(Kishko et al., 2011; Russell et al., 2011). The data obtained with b12 could not be compared between maternal and infant clones since most of them (15/17) were found resistant to this antibody. This low susceptibility to b12 is consistent with a recent phenotypic study that showed that only one out of 35 CRF01_AE Env-recombinant viruses was susceptible to b12 (Utachee et al., 2009). In contrast, all the transmitted viruses in the infants were highly sensitive to PG9 and PG16 (IC₅₀ <0.2 µg/mL and <0.4 µg/mL, respectively), significantly more sensitive than the maternal variants. This observation might indicate that PG9/PG16-sensitive variants would exhibit functional properties conferring a selective advantage for the mother-to-infant transmission. If confirmed in a larger population, it would suggest that PG9 and/or PG16 might be interesting for immunoprophylaxis of MTCT.

All maternal and infant clones were R5-tropic and similarly sensitive to TAK-779, a CCR5 antagonist. In contrast, both maternal and infant clones displayed a heterogeneous sensitivity to sCD4, but no statistically significant difference was observed between maternal and infant clones. This is consistent with a recent study performed on 5 clade B MIPs, in which all infant clones exhibited a wide range of sCD4 sensitivity, similar to that of maternal clones (Kishko et al., 2011). Our study also indicated that the viral infectivity levels in a single round of infection of both T2M-bl cells and PBMCs did not differ between maternal and infant variants despite a considerable variability in both variants. Working with pseudotyped viruses, we could not compare their replicative fitness in multiple rounds of infection and we cannot exclude a better replicative fitness of transmitted viruses. Previous studies performed on clade C MIPs have shown higher rates of replicative fitness of transmitted viruses than non-transmitted viruses despite, as we observed, a lack of difference between their infectivity levels (Kong et al., 2008; Zhang et al., 2010a). Similarly to susceptibility to sCD4 and infectivity levels, our data of Env processing efficiency and Env trimers incorporation levels did not seem to differ between maternal and infant clones. However, we observed that higher levels of Env trimers incorporation in virus particles correlated with increased infectivity levels. The most infectious virions were also those

that were the most resistant to sCD4, but surprisingly their resistance to sCD4 was not linked to a higher trimers density. Although several modes of sCD4-mediated inhibition were described, *i.e.* competitive inhibition, gp120 shedding (Moore et al., 1991; Orloff et al., 1993) or short-lived activated state of gp120 (Haim et al., 2009), these results suggested a decreased affinity of the envelope glycoprotein trimer of the most infectious virions for sCD4 and a possible biological advantage of virions expressing trimers adopting a conformation that better occludes the CD4 binding site. Together, these results suggested that the quantity of Env trimers rather than their affinity for the CD4 receptor, by facilitating the interaction with the CD4 receptor at the cell surface, modulates the infectivity of virions. Because all our pseudotyped viruses harbor the same transmembrane glycoprotein derived from NL4.3 prototype strain, the differences in Env incorporation must be attributed to the gp120 region only.

We previously observed a high degree of conservation of two PNGS in infant viruses, at positions N301 in V3 and N384 in C3, and hypothesized that they may confer a selective advantage for transmissibility of the virus to infants (Samleerat et al., 2008). We therefore compared the antigenic and functional properties of pseudotyped viruses according to the presence or not of N301 and/or N384. Despite the low number of functional viruses obtained, we could make some observations confirming that these two PNGS may play a role in resistance to autologous sera. This is consistent with previous studies that showed by *in vitro* studies that N301 was associated with a decrease in sensitivity of HIV-1 to neutralization by CD4BS antibodies (Koch et al., 2003; Malenbaum et al., 2000). However, PNGS at these two positions were not associated with infectivity or sensitivity to sCD4.

In conclusion, although limited in samples size, our study suggests that both the founder/transmitted viruses of the CRF01_AE clade present in infants and the variants present in their chronically infected mothers display a wide spectrum of biological properties, albeit the genetic bottleneck that occurred during transmission. Neither Env processing, Env incorporation efficiency, infectivity level, nor sensitivity to sCD4 or TAK-779 was associated with transmission. In other words, we did not find any specific property that would explain the selection of the founder/transmitted viruses, except sensitivity to PG9 and PG16. Maternal variants were less sensitive to neutralization by PG9 and PG16 than the founder/transmitted variants present in the infants. These data may suggest that the development of resistance to these antibody specificities could have some detrimental effect for the mother-to-child transmission of HIV-1. This observation may have implications for the development of prophylactic strategies to prevent MTCT.

Materials and methods

Study population

We selected samples from four mother–infant pairs (# 0377, 0858, 0978 and 1021) enrolled in the “Perinatal HIV Prevention Trial” cohort (Lallemant et al., 2000), that were described in a previous study (Samleerat et al., 2008). Env clones from these four pairs corresponded to a 1.2 kb fragment of the VI–V5 region covering almost the entire HIV-1 *env* gp120 gene (from upstream V1 to downstream V5) previously cloned in pCR2.1 vector (Invitrogen). Accession numbers are indicated in Table 2.

Construction of chimeric *env* genes

In order to obtain complete gp160 *env*, we constructed chimeric *env* genes in a NL4.3 backbone as described previously (Braibant et al., 2010). Briefly, the complete gp160 NL4.3 *env* gene was inserted into the *EcoRI* site of the pCR2.1 vector. Part of the *env* gene coding for V1 to V5 regions was extracted from this construct using *NdeI*

and *MfeI* restriction (New England BioLabs) and replaced by the corresponding gp120 sequence of interest excised from the pCR2.1 vector by digestion with the same enzymes. Chimeric *env* genes containing each gp120 sequence inserted in the NL4.3 backbone were then subcloned into the *EcoRI* site of the pCI expression vector (Promega).

Generation of *env*-pseudotyped viruses

Env-pseudotyped viruses were generated as described previously (Samleerat et al., 2009). 3.5×10^6 293T cells were cotransfected with 12 µg of each pCI-*env* plasmid and 8 µg of pNL4.3.LUC.R-E- (Connor et al., 1995), using phosphate calcium (Invitrogen). Viral supernatants were collected 72 h later, purified by filtration (0.45 µm filter) and stored as aliquots at -80°C . Viral infectivity was monitored by infection of 1×10^4 TZM-bl cells with 100 µL of serial 5-fold dilutions of the viral supernatants in quadruplicate in the presence of 30 µg/mL of DEAE-dextran. Infection levels were determined after 48 h, using the Bright Glo luciferase assay (Promega) and a Centro LB 960 luminometer (Berthold Technologies) to measure luciferase activity in cell lysates. Results with Relative Light Unit (RLU) values > 2.5 times the negative control (cells alone) were considered positive.

Cell culture

293T and U373-MAGI cell lines were grown at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum (FCS) and antibiotics (100 IU of penicillin and 100 µg/mL of streptomycin). U373-MAGI-CXCR4 and U373-MAGI-CCR5 cells were cultured in medium supplemented with 1 µg/mL of puromycin and 100 µg/mL of hygromycin B. TZM-bl cells were maintained in DMEM + pyruvate supplemented with 10% FCS, 50 µg/mL of gentamicin and 25 mM of HEPES (Platt et al., 1998; Wei et al., 2002). Frozen peripheral blood mononuclear cells (PBMC) from HIV-1 negative blood donors were treated with 5 µg/mL of phytohemagglutinin in RPMI 1640 medium supplemented with 10 ng/mL of interleukin-2 (Roche), 20% FCS and antibiotics for 3 days. They were then washed and maintained in RPMI 1640 medium supplemented with interleukin-2, 20% FCS and antibiotics.

Determination of co-receptor usage

Co-receptor usage was determined using the U373-MAGI cell lines. U373-MAGI cells expressed the CD4 receptor with either the CXCR4 co-receptor (U373-MAGI-CXCR4) or the CCR5 co-receptor (U373-MAGI-CCR5). 1.5×10^4 cells were plated the day prior infection. Cells were infected with 25 µL of a normalized p24 amount (10 ng) of pseudotyped viruses for 2 h at 37°C . Then, 175 µL of DMEM supplemented with 20 µg/mL of DEAE-dextran and 5% FCS were added. 48 h after infection, the luciferase activity was measured and the viral tropism was determined.

Neutralization and inhibition assay

Sensitivity to autologous and heterologous plasmas, mAbs b12, PG9 and PG16, and to sCD4 and TAK-779 entry inhibitors, were assessed in duplicate in TZM-bl cells. After titration, pseudotyped virus stocks were diluted to obtain 1000 TCID₅₀/mL in growth medium. Aliquots of 25 µL were then incubated for 1 h at 37°C with 75 µL of either two-fold serial dilutions of heat-inactivated serum (1:20 to 1:10240), or b12 (50 µg/mL to 0.1 µg/mL; Polymun Scientific), or sCD4 (10 µg/mL to 0.02 µg/mL; NIBSC), or three-fold serial dilutions of PG9 and PG16 (10 µg/mL to 0.005 µg/mL; IAVI). Then 1×10^4 TZM-bl cells were added to the virus/serum mixture in the presence of 30 µg/mL of DEAE-dextran. Luciferase activity was measured 48 h after infection as described above. Results were expressed as mean values. IC₅₀ values

were defined as the reciprocal of the serum dilution or antibody concentration required to reduce RLU by 50%.

For TAK-779 inhibition, 8×10^3 TZM-bl cells per wells were prepared the day prior infection. Cells were first treated for 1 h with 75 μ L of two-fold serial dilutions of TAK-779 (0.2 μ g/mL to 0.0004 μ g/mL; NIH AIDS Research and Reference Reagent Program) before adding 25 μ L of pseudotyped viruses normalized to 1000 TCID₅₀/mL. 100 μ L of DMEM medium supplemented with 30 μ g/mL DEAE-dextran were then added to cells. Luciferase activity was measured 48 h after infection as described above.

Viral infectivity in TZM-bl cells and peripheral blood mononuclear cells

Viral infectivity was determined in quadruplicate in TZM-bl and PBMC cells. 25 μ L of virus stock normalized at 10 ng p24 were added to 75 μ L of culture medium. 1×10^4 TZM-bl cells or 1×10^5 PBMC cells were added to viruses in the presence of 30 μ g/mL DEAE-dextran. Luciferase activity was measured in the cells lysates 48 h after infection. Results were expressed as a ratio of mean RLU values obtained for each virus compared to mean RLU values obtained with the NL4.3 virus.

Processing and incorporation of envelope glycoproteins

Viral supernatants were overlaid on a 20% sucrose cushion, and viral particles were pelleted at 87,000 g for 1.5 h at 4 °C. Viral pellets were solubilized for 5 min in 100 μ L of phosphate buffered saline (PBS) supplemented with 1% Triton X-100 and an antiprotease cocktail (aprotinin 2 μ g/mL, leupeptin 2 μ g/mL, phenylmethanesulfonylfluoride 1 mM). P24 antigen content was determined by ELISA (INNOTEST® HIV Antigen mAb; Innogenetics) and aliquots of the resuspended pellets were stored as at –80 °C until used. Glycoproteins analyses were performed by western blotting after SDS-PAGE and Blue-Native-PAGE (BN-PAGE).

SDS-PAGE

Samples containing 100 ng p24 were boiled for 5 min in Laemmli sample buffer in the presence of dithiothreitol, and were separated by electrophoresis in a SDS-10% polyacrylamide gel. The proteins were then transferred onto a nitrocellulose membrane for 1.5 h at 100 V. Blots were probed for gp120 and p24 with specific goat polyclonal antibodies (1/1000; AbD Serotec) in Tris buffer saline (TBS) containing 2% nonfat milk by incubation overnight at 4 °C, followed after washings by incubation with a horseradish peroxidase (HRP) conjugated anti-goat-IgG from rabbit (1/5000; Jackson ImmunoResearch) for 1 h at room temperature. Immunoblots were developed using a luminol-based enhanced chemiluminescence substrate (ECL Plus Western Blotting Detection System; Amersham). Env gp120 and gp160, as well as p24 proteins were quantified using the Bio-1D++ analysis software (Vilber Lourmat; Deutschland GmbH).

BN-PAGE

To analyze Env gp under native conditions, we used a modified BN-PAGE protocol (Binley et al., 2010; Crooks et al., 2007; Moore et al., 2006). Solubilized virions were normalized for p24 amount (100 ng). 2× native sample buffer (125 mM Tris-HCl pH 6.8, 40% glycerol, 0.1% Coomassie blue G250) was added to samples prior to loading onto a 4 to 15% Mini-PROTEAN® TGX™ gel (BioRad). High molecular weight calibration Kit for native electrophoresis (Amersham) was used for size determination. Samples were separated at 4 °C for 2.5 h at 100 V with TG 1X (25 mM Tris–192 mM Glycine) containing 0.002% Coomassie blue as cathode buffer and TG 1× as anode buffer. The gel was then transferred onto a polyvinylidene difluoride (PVDF) membrane for 1.5 h at 100 V. PVDF membranes were destained with a 30% methanol/10% acetic acid solution, then with 100% methanol. Blots were probed with mAbs 2 F5 and 4E10 (1 μ g/mL; Polymun)

in TBS containing 2% nonfat milk by incubation overnight at 4 °C, followed after washings by incubation with a horseradish peroxidase (HRP) conjugated anti-human-IgG from goat (1/5000; Jackson ImmunoResearch) for 1 h at room temperature. Immunoblots were developed with a luminol-based enhanced chemiluminescence substrate (ECL Plus Western Blotting Detection System; Amersham). Env trimers were quantified using the Bio-1D++ analysis software (Vilber Lourmat; Deutschland GmbH).

Statistical analyses

A mixed-model approach was used to compare each biological property between maternal and infant clones. This is the most suitable technique for estimating differences according to the origin (maternal or infant) of the clone, with allowance for the correlation structure of properties within each MIP and for differences in the number of observations within each MIP. For each property, the origin of the clone was considered as the fixed effect and MIP's effect was assumed to be random. In cases in which the IC₅₀ values were <20, the midpoint value between 0 and 20, 10, was assigned. When the IC₅₀ values were >10, a value of 10 was assigned. Significance was reported when $P \leq 0.05$. Correlations between two biological properties were examined with the Spearman's correlation test. Biological properties according to PNGS at positions N301 or N384 were compared with the Mann–Whitney test.

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