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Development of a tier 1 R5 clade C simian–human immunodeficiency virus as a tool to test neutralizing antibody-based immunoprophylaxis

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Abstract

Background—While some recently transmitted HIV clade C (HIV-C) strains exhibited tier 1 neutralization phenotypes, most were tier 2 strains (*J Virol* 2010; 84:1439). Because induction of neutralizing antibodies (nAbs) through vaccination against tier 2 viruses has proven difficult, we have generated a tier 1, clade C simian–human immunodeficiency virus (SHIV-C) to permit efficacy testing of candidate AIDS vaccines against tier 1 viruses.

Methods—SHIV-1157ipEL was created by swapping *env* of a late-stage virus with that of a tier 1, early form.

Results—After adaptation to rhesus macaques (RM), passaged SHIV-1157ipEL-p replicated vigorously *in vitro* and *in vivo* while maintaining R5 tropism. The virus was reproducibly transmissible intrarectally. Phylogenetically, SHIV-1157ipEL-p Env clustered with HIV-C sequences. All RM chronically infected with SHIV-1157ipEL-p developed high nAb titers against autologous as well as heterologous tier 1 strains.

Conclusions—SHIV-1157ipEL-p was reproducibly transmitted in RM, induced cross-clade nAbs, and represents a tool to evaluate anti-HIV-C nAb responses in primates.

Keywords

clade C SHIV; neutralization sensitive; R5 SHIV; tier 1; vaccine development

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Introduction

Human immunodeficiency virus type 1 (HIV-1) is classified into several clades based upon genetic diversity. HIV-1 clade C (HIV-C) is the major subtype and accounts for 56% of all HIV-1/AIDS cases worldwide. It is prevalent in Sub-Saharan Africa, Asia, including India and China, and Brazil (http://www.unaids.org). The epidemiological data suggest a critical need for vaccines to stop the continued spread of HIV-C across the globe.

More than 90% of all HIV-1 transmissions occur mucosally, and almost all of these are initiated by R5 viruses, even when the source persons have mixed infections [16]. Therefore, the availability of a primate model that reflects the prominent biologic features of HIV-1 transmission among humans will enhance our understanding of HIV-1 pathogenesis and facilitate the development of an effective vaccine.

We have generated a series of R5-tropic clade C simian–human immunodeficiency viruses (SHIV-Cs), among them SHIV-1157ipd3N4 [21] and SHIV-2873Nip [19]. The neutralization sensitivity of the latter viruses is typical of primary isolates (tier 2 profiles, indicating only moderate neutralization sensitivity) [18]. Both viruses have caused disease progression to AIDS in several monkeys while maintaining strict R5 tropism [4, 12].

Here, we report the adaptation and viral characteristics of SHIV-1157ipEL-p, a typical tier 1 virus that not only induced high levels of neutralizing antibodies (nAbs) against the autologous virus, but also against other tier 1 strains, including those of different clades.

Materials and methods

Animals

Colony-derived rhesus macaques (RM) of Indian origin were housed at the Yerkes National Primate Research Center (YNPRC, Emory University, Atlanta, GA) according to National Institutes of Health guidelines on the care and use of laboratory animals. YNPRC facilities are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Animal experiments were approved by the Animal Care and Use Committees of Emory University and the Dana-Farber Cancer Institute.

Generation of the infectious molecular clone, SHIV-1157ipEL

SHIV-1157ipEL was constructed using the late-stage virus, SHIV-1157ipd3N4 [21] as the backbone. The *env* gene of the late virus was exchanged with the early form, which was excised from the R5 SHIV-1157ip [12]. Details of the SHIV-1157ipEL construction and *in vitro* evaluation are described elsewhere [20].

The initial virus stock of the parental infectious molecular clone, SHIV-1157ipEL, was generated by transfection of 293T cells and harvesting of cell-free supernatant, which was used to infect RM peripheral blood mononuclear cells (PBMC). This initial PBMC-derived stock was used to determine coreceptor usage, ability to replicate in PBMC of randomly selected RM blood donors, and neutralization sensitivity as was described elsewhere [20].

Intravenous and intrarectal challenges

The parental SHIV-1157ipEL stock was inoculated intravenously (i.v.) into RM REk-11. After this animal was confirmed virus positive by reverse-transcriptase polymerase chain reaction (RT-PCR), 10 ml of blood from REk-11 was transferred i.v. to the next recipient RM (RIj-11) at week 2 post-inoculation. Virus was passaged at the time of peak viremia (week 2) according to previously published protocols [12, 19] through a total of four RM. All animals were monitored for viral loads, T-cell subsets, and antibody responses. A stock

of the passaged virus, SHIV-1157ipEL-p, was generated by isolating virus from the fourth recipient and expansion in RM PBMC.

To test the mucosal transmissibility of SHIV-1157ipEL-p and to establish a 5× low-dose challenge dose, six animals received repeated weekly low-dose intrarectal (i.r.) inoculations (up to a maximum of five). For intrarectal inoculations, a previously published protocol was used [4]. Monkeys that remained either aviremic or had only transient, low-level viremia (< 10^4 copies/ml) at the 2-week time point after the fifth low-dose SHIV-1157ipEL-p exposure were given a single high-dose i.r. challenge [approximately nine 50% animal infectious doses (AID_{50})]. Blood was collected at 0, 1, 2, 4, 8, and 12 weeks post-inoculation and monthly thereafter to determine viral RNA (vRNA) loads and T-cell subsets.

Measurement of plasma vRNA levels

Plasma vRNA was isolated using the QiaAmp Viral RNA Mini-kit (Qiagen, Valencia, CA, USA) and vRNA levels were measured by quantitative RT-PCR for SIV *gag* sequences [11]. Assay sensitivity was determined to be 50 vRNA copies/ml. We also used primers/probes for SIV *gag* according to Cline et al. [5].

Sequencing and phylogenetic analysis

Chromosomal DNA was extracted from PBMC of the last RM during the adaptation process using a DNA-zol genomic DNA isolation kit (Molecular Research Center Inc., Cincinnati, OH, USA). Using the following pair of primers, PCR was carried out under endpoint dilution: forward (5′-AGTCTATTATGGGGTACCTGTATGGAAAGAAGCA-3′) and reverse (5′-TCCCAGATAAGTGCCAAGGATCCGTTCACTAATC-3′); the amplified fragment was cloned into the *KpnI* and *BamHI* sites of a pcDNA6/myc-His B vector for sequencing. DNA sequencing was performed for five randomly selected clones encoding an e*nv* gene. The evolutionary history was deduced by use of the neighbor-joining method [17]. The optimal tree with the sum of branch length = 1.03331016 is depicted. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method [23] and are in the units of the number of base substitutions per site. The analysis involved 34 amino acid sequences. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 2063 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 [22].

Neutralization assays

Neutralization titers of sera obtained from RM with prolonged chronic SHIV-1157ipEL-p infection were measured using the TZM-bl reporter cell line-based neutralization assay as described previously [13, 14]. TZM-bl cells (also called JC53-bl [6] cells obtained from NIH AIDS Research and Reference Reagent Program (ARRRP) stably express CD4 and CCR5 as well as luciferase and β-galactosidase under the control of the HIV-1 long terminal repeat.

PBMC-based neutralization assays were performed using polymyxin B (15 μg/ml) throughout the assay period to rule out blocking of virus replication via chemokines induced by lipopolysaccharide [7, 25]. Human PBMC were stimulated overnight with phytohemagglutinin (5 μg/ml), followed by the addition of interleukin-2 (IL-2) (20 U/ml) for 48 hours. PBMC were washed twice and added to wells at 5×10^5/well in 96-well plates. Heat-inactivated sera at various dilutions were incubated with a pre-determined 50% tissue culture infectious dose ($TCID_{50}$) of the virus for 1 hour and added to cells to yield a final volume of 250 μl and left for 3 days. As controls, virus was incubated with medium alone

(negative control) or with cells alone (positive control to determine 100% virus production). Starting from day 3 after virus addition, half of the medium was replaced daily with fresh medium (containing IL-2 and polymyxin B) up to day 10. Supernatants were harvested daily, assayed for p27 levels, and the percent neutralization was measured on the culture day showing a linear phase of increase with p27 levels ranging between 8 and 15 ng/ml in the virus + cells control wells.

Results

Construction of SHIV-1157ipEL encoding a tier 1, neutralization-sensitive *env*

The *env* gene of the tier 1 neutralization-sensitive SHIV-1157ip was PCR-amplified from DNA isolated from cocultured PBMC of a SHIV-1157ip-infected RM [12]. SHIV-1157ipEL was constructed by inserting the early *env* gene from SHIV-1157ip into the backbone of the late-stage virus, SHIV-1157ipd3N4 [21]. Cell-free virus for initial characterization was generated by transfecting the molecular clone into 293T cells followed by a brief expansion in RM PBMC and tested for replication kinetics as described in the following.

Replication of SHIV-1157ipEL and SHIV-1157ipEL-p in RM PBMC and coreceptor usage

To test the replication competence of the new parental SHIV construct, SHIV-1157ipEL, we randomly selected nine naïve RM blood donors. The new virus was found to replicate in PBMC of only eight of the nine donors tested (Fig. 1A), with peak p27 production detected in the tissue culture supernatants between days 10 and 12. These data implied that the new SHIV strain needed to undergo further adaptation for optimal replication in unselected RM PBMC.

After rapid passage through 4 RM, we re-isolated the adapted virus, termed SHIV-1157ipEL-p. Next, we retested its ability to replicate in PBMC of those RM, which previously had failed to replicate SHIV-1157ipEL, as well as in PBMC of other randomly selected donors. The passaged virus clearly replicated vigorously in all PBMC cultures tested (Fig. 1B), indicating its adaptation to the new host species.

We also assessed the coreceptor usage of SHIV-1157ipEL and SHIV-1157ipEL-p. As described previously [20], we observed productive infection only in U87.CD4.CCR5 cells and none in any cell line lacking CCR5, including CEMx174-GFP, U87.CD4, U87.CD4.CCR1, U87.CD4.CCR2, U87.CD4.CCR3, U87.CD4.CXCR4, GHOST-BOB, and GHOST-BONZO cells, suggesting that both viruses exclusively use CCR5 as a coreceptor for entry.

Neutralization tiers of SHIV-1157ipEL and SHIV-1157ipEL-p

Next, we tested the neutralization tiers of the parental infectious molecular clone and the passaged progeny virus, a biologic isolate, using the TZM-bl assay (Table 1). The rapid passage through RM at peak viremia had not appreciably altered the high neutralization sensitivity of the passaged virus. Thus, our adaptation strategy had avoided selection of a neutralization escape virus. In contrast, the late form, SHIV-1157ipd3N4, was considerably less sensitive to neutralization and exhibited a typical tier 2 profile.

Phylogenetic analysis

All envelopes of the 1157i series of SHIVs clustered with HIV-C (Fig. 2). The close proximity of the SHIV-1157i, SHIV-1157ip, and SHIV-1157ipEL-p as well as SHIV-1157ipd3N4 *env* genes on the phylogenetic tree reflects the same ancestral origin for this series of SHIVs (Fig. 2). All the newly generated clones of SHIV-1157ipEL-p were clustered together with minimal diversity (Fig. 2). Interestingly, the tier 2 envelope of the

late virus, SHIV-1157ipd3N4, had a greater distance from the closely clustered tier 1 envelopes of SHIV-1157i, SHIV-1157ip, and SHIV-1157ipEL-p. We also found that the HIV-C envelopes contained in our two different sets of R5 SHIV-Cs (the 1157i and 2873i series) were more closely related to each other than to the other HIV-C Env sequences. This is probably due to the fact that the pediatric HIV-C strains (HIV1157i and HIV2873i) used to isolate the *env* genes for SHIV construction were derived from the same cohort of HIV-C-infected infants in Lusaka, Zambia. The divergence of two pediatric HIV-C envelopes thus reflects envelope sequence divergence in a local community.

SHIV-1157ipEL-p inoculations in RM

To demonstrate reliable mucosal transmissibility of SHIV-1157ipEL-p, we summarized the data from a total of 31 RM inoculated by the i.r. route (Table 2). All RM were of Indian origin. Twenty juvenile/adult RM received weekly low-dose challenges (up to a maximum of five inoculations) with virus doses ranging between 5000 and 10,000 $TCID_{50}$. Another five juvenile/adult RM as well as six infants were given a single high-dose i.r. challenge. SHIV-1157ipEL-p replicated vigorously in all i.r. challenged RM with high peak viremia levels ranging from 2×10^5 to 4.1×10^7 RNA copies/ml (Fig. 3B; Table 2). We have also listed the peak vRNA levels of the four RM used in the initial adaptation of the parental SHIV-1157ipEL (Fig. 3A; Table 2). These animals were inoculated i.v. with infected blood. Chronically infected RM are currently being followed prospectively for signs of disease progression.

Depletion of CD4 T cells during acute viremia

SHIV-1157ipEL-p-infected monkeys (n = 8) were followed for the natural course of infection and disease. Approximately 60–110 weeks post-inoculation, these monkeys have maintained absolute $CD4^+$ T cells in peripheral blood of >500 cells/μl (Fig. 3C). However, we observed persistently low (<10%) peripheral blood $CD4^+$ memory T cells (assessed by $CD4^+CD29^+$ double staining) in all infected animals (data not shown). We also assessed the acute pathogenicity of SHIV-1157ipEL-p by evaluating the absolute cell counts or percentage of $CD4^+$ T cells in blood as well as in rectal and lymph node lymphocyte populations (2–12 weeks post-inoculation). As shown previously [20], statistically significant depletion of $CD4^+$ T cells was observed in blood, lymph nodes, and the gut in all infected RM when compared to uninfected RM.

Cross-clade nAbs induced during chronic SHIV-1157ipEL-p infection

Plasma or serum samples from SHIV-1157ipEL-p-infected animals with long-term infection (10 months to 2 years) were tested for their ability to neutralize autologous or heterologous SHIVs in TZM-bl and PBMC-based neutralization assays. All animals tested developed nAbs against the autologous virus as well as against heterologous tier 1 viruses (Table 3); in some cases, titers were very high. In contrast, the tier 2 virus SHIV-2873Nip was not neutralized. PBMC-based assays yielded similar results, although generally higher titers were observed (data not shown).

Discussion

We have described some of the biologic characteristics of the newly generated R5 SHIV-1157ipEL-p: (1) it carries *env* of an early, relatively recently transmitted pediatric HIV-C from Zambia; (2) it was highly replication competent in PBMC cultures of non-selected RM and induced high peak vRNA loads in all animals tested; (3) its envelope was shown to cluster with HIV-C Envs; and (4) it is highly sensitive to neutralization and exhibits a tier 1 profile.

SHIV-1157ipEL-p carries the tier 1 envelope isolated from a 6-month old Zambian infant [12]. Although several other SHIV strains encoding HIV-C envelopes have been created, they were unable to replicate in RM PBMC ($SHIV_{CHN19}$) [3], could not be re-isolated after adaptation ($SHIV_{MJ4}$ and SHIV-XJ02170) [15, 26], had dual tropism (SHIV-MCGP1.3) [2], or are relatively difficult to neutralize with tier 2 profiles (SHIV-1157ipd3N4 and SHIV-2873Nip) [19, 21]. In contrast, the newly created R5 SHIV-1157ipEL-p is highly sensitive to neutralization with a tier 1 profile. It not only replicated well in PBMC of all RM donors tested, but also showed robust replication *in vivo* with high peak vRNA loads after mucosal challenge.

After adaptation, genetic analysis of the SHIV-1157ipEL-p envelope showed only a few point mutations when compared to that of the parental chimera SHIV-1157ipEL, indicating that our rapid, every 2-week passage strategy resulted in only minor amino acid changes. Not surprisingly, the tier 1 neutralization profile was maintained after adaptation.

Based initially on the *env* gene of the recently transmitted pediatric HIV1157i, we have created a matched pair of tier 1/tier 2 R5 SHIV-Cs, namely SHIV-1157ipEL-p and SHIV-1157ipd3N4. The tier 2 envelope in our late SHIV-1157ipd3N4 strain evolved from the early, highly neutralization-sensitive form. Another pair of tier 1/tier 2 R5 SHIVs has been described in the literature, namely $SHIV_{SF162P4}$/$SHIV_{SF162P3}$ [1, 8, 24]. The envelopes of these clade B SHIVs are based upon HIV_{SF162}. Surprisingly, the tier 1 $SHIV_{SF162P4}$ arose in a macaque from a later passage compared to the tier 2 $SHIV_{SF162P3}$ [1]. Interestingly, two different X4 viruses emerged from $SHIV_{SF162P3N}$-infected animals [9, 10], and data obtained from these monkeys will provide important clues for factors associated with *in vivo* coreceptor switch. We have not yet observed any coreceptor switch in our SHIV-C-infected macaques – regardless of viral neutralization tier. We plan to follow monkeys with chronic SHIV-C viremia prospectively for evidence of expanded coreceptor usage.

To date, SHIV-1157ipEL-p has not yet induced AIDS given that follow-up times have been relatively short. However, this new tier 1 virus was constructed using components derived from pathogenic viruses: the backbone originated from SHIV-1157ipd3N4 [21] and the *env* gene from SHIV-1157ip [12]. Both of these viruses caused AIDS in RM independently [4, 12], and we therefore expect a similar tendency in the case of SHIV-1157ipEL-p. Prolonged, prospective follow-up will reveal the pathogenic potential of SHIV-1157ipEL-p.

In summary, we showed the adaptation and replication kinetics of the tier 1 virus, SHIV-1157ipEL-p, *in vitro* and *in vivo*. Of note, sera from all animals infected for extended time periods with this virus contained cross-clade nAbs. We suggest that this new virus represents a biologically relevant tool to evaluate anti-HIV-C nAb responses in primates and Env evolution under the pressure of autologous nAb responses. Given the robust replication in Indian RM, this tier 1 virus and/or SHIV-1157ipd3N4 can be used to test the efficacy of nAb-response-based vaccine candidates. Likewise, the new SHIV-1157ipEL-p or its tier 2 counterpart – depending on *in vitro* neutralization results – can be used to assess the ability of novel human neutralization monoclonal antibodies to provide protection via passive immunization.

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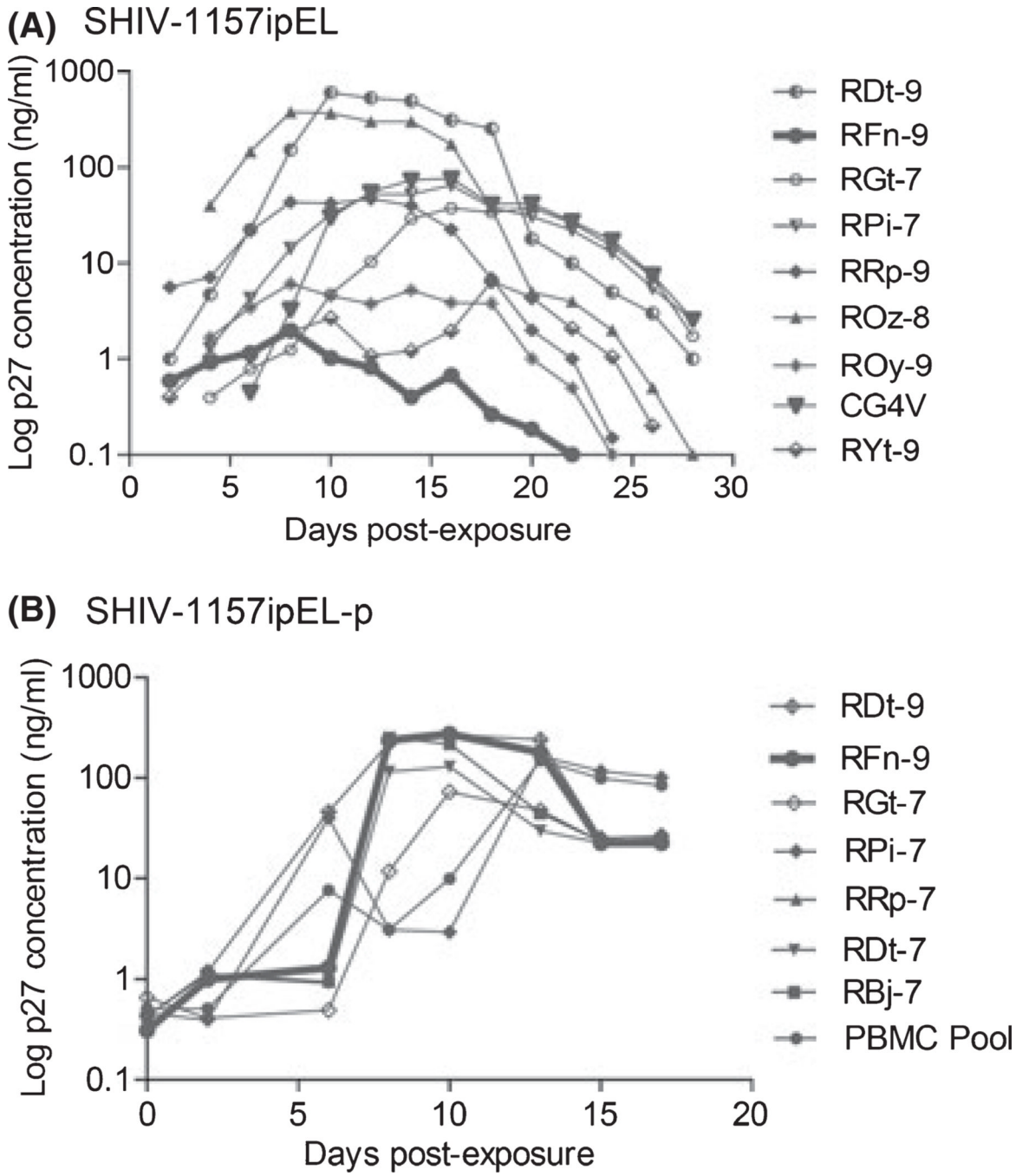

Fig. 1.

Replication of SHIV-1157ipEL and SHIV-1157ipEL-p in rhesus macaque (RM) PBMC. (A) PBMC from nine randomly selected naïve RM donors were stimulated with concanavalin A (ConA) and exposed to SHIV-1157ipEL-containing supernatant (Methods). The PBMC of RM RFn-9 did not support the replication of parental SHIV-1157ipEL (thick line). (B) PBMC from random donors were stimulated with Con A and exposed to passaged virus, SHIV-1157ipEL-p. Supernatants were harvested and p27 levels were measured at the time points indicated. Thick line, PBMC of RM RFn-9 (previously unable to support replication of parental virus, see under A) now yielded high levels of p27 production, indicating viral

adaptation to the new host species. SHIV, simian–human immunodeficiency virus; PBMC, peripheral blood mononuclear cells.

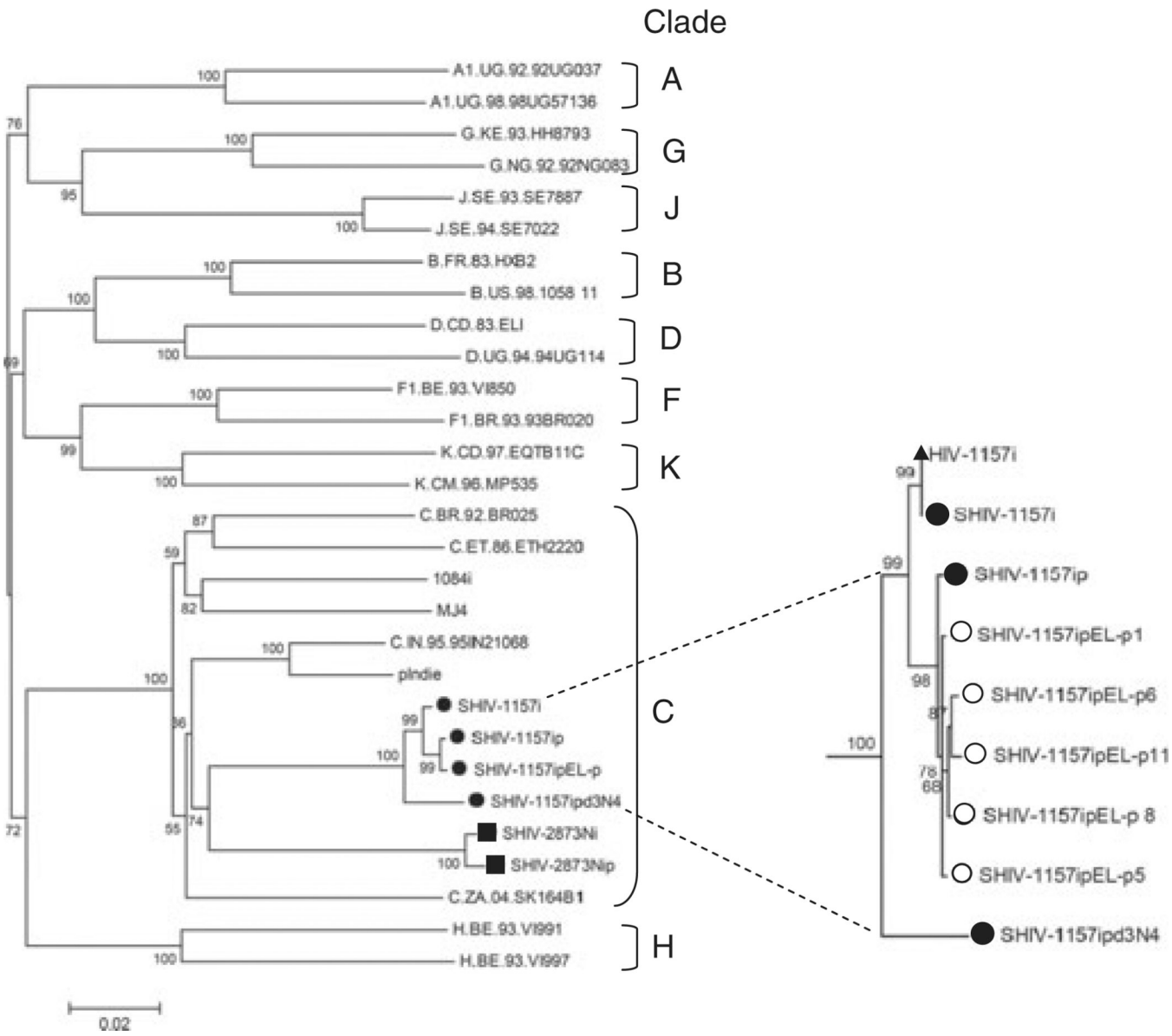

Fig. 2.

Phylogenetic tree construction. Phylogenetic tree showing the relationship between SHIV-1157i, SHIV-1157ip, SHIV-1157ipEL-p, and SHIV-1157ipd3N4 Env sequences and those of other primary strains of HIV. Phylogenetic trees were constructed from full-length Env sequences by using MEGA4. Major clades of HIV group M were used as reference sequences; Env sequences from SHIV-2873Nip and HIV1084i were also included, as SHIV-2873Nip and HIV1084i *env* were genes derived from the same cohort of HIV-C-infected infants in Lusaka, Zambia. The scale bar indicates the genetic distance along the horizontal branches, and the numbers at the nodes are bootstrap values. The insert represents the phylogenetic relationship between HIV-1157i and the SHIV series related to the *env* gene of the latter. The open circles denote five individual SHIV-1157ipEL-p sequences obtained through end-point PCR cloning that were used to arrive at the consensus sequence of SHIV-1157ipEL-p. Closed circles represent the consensus sequence of each of the viruses as noted in the figure. The closed triangle represents the parental sequence of HIV-1157i

that was used to generate the initial SHIV clone. SHIV, simian–human immunodeficiency virus; HIV-C, HIV clade C.

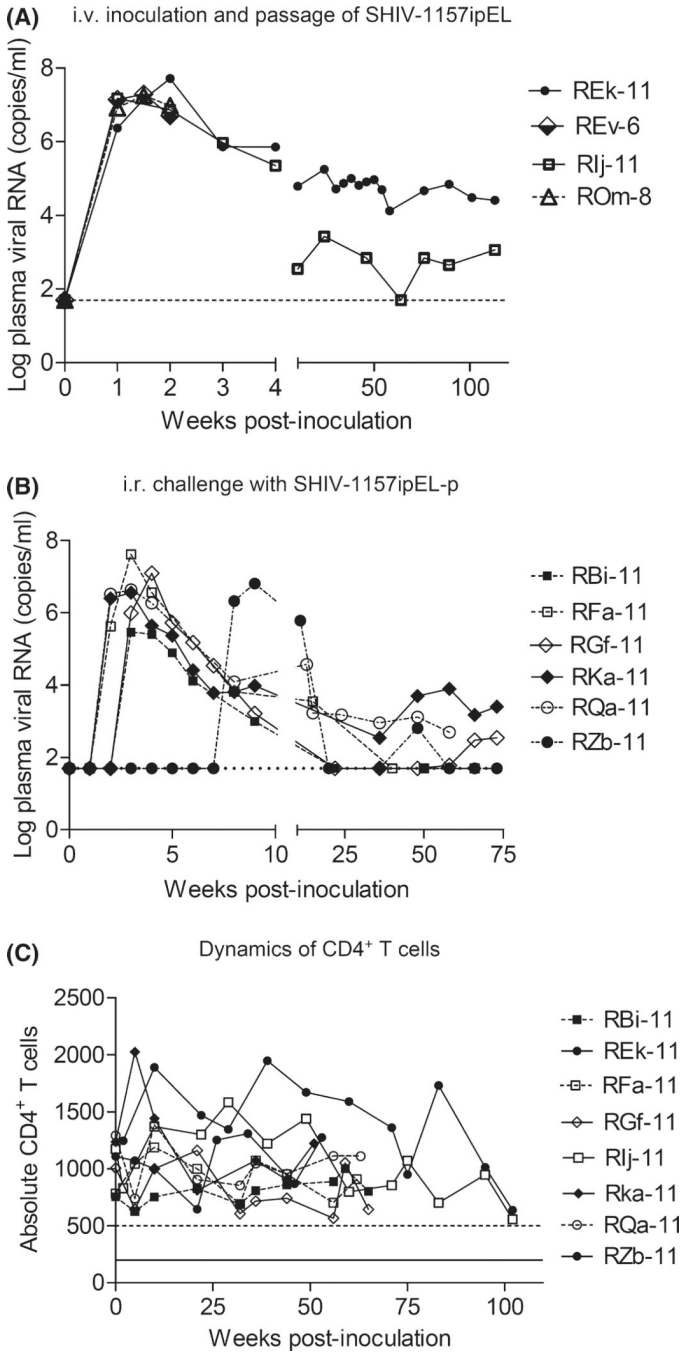

Fig. 3.

(A) Intravenous inoculation of SHIV-1157ipEL: monkey REk-11 was inoculated i.v. with 10 ml of PBMC-grown virus. This was followed by rapid passage at peak viremia (week 2 post-inoculation) through three additional RM using serial blood transfer (10 ml each). Viral RNA (vRNA) levels were measured at time points indicated. (B) Intrarectal inoculation of SHIV-1157ipEL-p in six monkeys, using limited weekly low-dose inoculations (maximally five). RM RZb-11 remained uninfected at week 2 after the fifth exposure and was given a single high-dose of SHIV-1157ipEL-p. vRNA levels were measured at the time points indicated. The horizontal dotted line indicates the lower limit of detection (<50 vRNA copies/ml). (C) Absolute $CD4^+$ T-cell counts of RM infected with SHIV-1157ipEL-p.

SHIV, simian–human immunodeficiency virus; RM, rhesus macaques; PBMC, peripheral blood mononuclear cells.

Table 1

Sensitivity of SHIV-C strains to soluble CD4, human nmAbs, and serum samples

	IC_{50} (μg/ml) in TZM-bl cells[1]						IC_{50} (reciprocal serum dilution) in TZM-bl cells[1]							
SHIV-C strain	**sCD4**	**IgG1b12**	**2G12**	**2F5**	**4E10**	**HIVIG**	**BB47**	**BB55**	**BB68**	**BB75**	**BB80**	**BB81**	**BB87**	**Tier**
SHIV-1157ipEL (parental infectious mol. clone with early *env*)	1.7	0.7	>25	>25	>25	88.6	4274	3286	666	1245	1822	691	3278	1
SHIV-1157ipEL-p (passaged biologic isolate with early *env*)	0.5	1.4	>25	>25	>25	150.8	1193	1792	653	824	1135	427	2707	1
SHIV-1157ipd3N4[2] (infectious mol. clone with late *env*)	0.4	7.0	>25	>25	>25	1160	105	131	86	79	72	47	260	2

SHIV, simian–human immunodeficiency virus.

Spectrum of neutralization sensitivity of R5 SHIV strains encoding HIV clade B or C *env*.

[1]Values represent the concentration (μg/ml for soluble CD4 (sCD4) and human nmAbs IgG1b12, 2G12, 2F5, 4E10, or HIVIG) or the dilution (for serum samples) at which relative luciferase units (RLU) were reduced 50% compared to virus control wells. BB47, BB55, BB68, BB75, BB80, BB81, and BB87 are serum samples from individuals infected with HIV-1 clade C. HIVIG, polyclonal high-titer anti-HIV Ig preparation.

[2]Data previously published and shown here as reference [19]; given as basis of comparison.

Table 2

SHIV-1157ipEL-p route of challenge and mean peak viral RNA (vRNA) loads

Age	No. of macaques	Route of exposure	Dose	Mean peak vRNA (copies/ml) × 10^6
Juvenile/adult	4	i.v. (infected blood in 3 out of 4 RM)	High	26.2 ± 17.5
	6	i.r.	High	3.0 ± 2.1
	19	i.r.	5× low	5.4 ± 9.5
Infants	6	i.r.	High	6.8 ± 3.9

SHIV, simian–human immunodeficiency virus.

Table 3

Neutralization titers of sera from SHIV-1157ipEL-p-challenged rhesus macaques

	Titers (plasma dilution at 50% neutralization in TZM-bl cells)				
	Homologous clade C (tier 1)	Heterologous clade C		Heterologous clade B	
Animal no.	SHIV-1157ipEL-p	SHIV-2873Nip (tier 2)	MW965.5 (tier 1)	SHIV-Bo159N4[1]	$SHIV_{SF162P4}$
RBi-11	128	<20	1498	49	83
REk-11	3690	<20	>43,740	9640	7615
RFa-11	1038	<20	8019	160	271
RGf-11	695	<20	4753	55	335
RIj-11	1985	<20	24,586	220	348
RKa-11	779	<20	8415	707	353
RQa-11	403	<20	3589	71	198
RZb-11	243	<20	1571	58	92

SHIV, simian–human immunodeficiency virus.

[1] This tier 1 R5 SHIV strain is of clade B origin and encodes extra NF-κB sites in the long terminal repeats (Siddappa et al., unpublished data).