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Derivation of non-infectious envelope proteins from virions isolated from plasma negative for HIV antibodies

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Abstract

Natural membrane-bound HIV-1 envelope proteins (mHIVenv) could be used to produce an effective subunit vaccine against HIV infection, akin to effective vaccination against HBV infection using the hepatitis B surface antigen. The quaternary structure of mHIVenv is postulated to elicit broadly neutralizing antibodies protective against HIV-1 transmission. The founder virus transmitted to infected individuals during acute HIV-1 infection is genetically homogeneous and restricted to CCR5-tropic phenotype. Therefore, isolates of plasma-derived HIV-1 (PHIV) from infected blood donors while negative for antibodies to HIV proteins were selected for expansion in primary lymphocytes as an optimized cell substrate (OCS). Virions in the culture supernatants were purified by removing contaminating microvesicles using immunomagnetic beads coated with anti-CD45. Membrane cholesterol was extracted from purified virions with beta-cyclodextrin to permeabilize them and expel p24, RT and viral RNA, and permit protease-free Benzonase to hydrolyze the residual viral/host DNA/RNA without loss of gp120. The resultant mHIVenv, containing gp120 bound to native gp41 in immunoreactive form, was free from infectivity in vitro in co-cultures with OCS and in vivo after inoculating SCID-hu Thy/Liv mice. These data should help development of mHIVenv as a virally safe immunogen and enable preparation of polyclonal hyper-immune globulins for immunoprophylaxis against HIV-1 infection.

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Keywords

HIV inactivation; mHIVenv proteins; SCID-hu Thy/Liv mouse model; mHIVenv subunit vaccine; mHIVenv immune globulins

1. Introduction

With 33.4 million people living with HIV-1 infection and 2.7 million newly infected each year, the development of a safe and effective vaccine to prevent the spread of HIV infection remains a paramount public health objective [1]. Synthetic vaccines for HIV, using cloned envelope proteins (gp160 and gp120) or cloned viral genes inserted in a variety of vectors, have not elicited broadly neutralizing antibodies (bNAb). This shortcoming likely explains their failure to protect against HIV transmission [2]. One way to overcome this difficulty is to construct a vaccine based on transmitted pathogen that has been rendered safe and incapable of producing disease, yet retains the surface molecular organization of the natural agent [3]. This concept is best exemplified by the first vaccine licensed for preventing hepatitis B virus (HBV) infection with 20 nm particles of natural hepatitis B surface antigen (HBsAg) isolated from HBV-infected plasma [4]. The antigenicity and immunogenicity of HBsAg is conformationally determined by the disulfide bonds formed by the dimeric envelope proteins, which are as immunogenic as the native particles formed by assembly of 49 kD subunits in membrane lipid bilayer [4–7]. When the 49kD subunits were reduced with 2-mercaptoethanol, they dissociated into 22 kD and 27 kD poly-peptides with a drastic loss of antigenicity and immunogenicity [7]. The protection afforded by primary immunization with plasma-derived hepatitis B vaccine during childhood and adulthood lasts at least 22 years, and booster doses are not needed [8]. Therefore, HBsAg may serve as a model for HIV vaccine development.

The quaternary structures of conformationally conserved trimeric heteroduplex subunits of HIV envelope proteins bound to the virion membrane are considered necessary for eliciting bNAb [9]. An effective HIV vaccine must target the transmitted virus; importantly this virus can differ from the virus that evolves soon after transmission. We hypothesize that subunits of membrane-bound HIV envelope proteins (mHIVenv), isolated from inactivated virions of representative genetic subtypes transmitted in the world, will be particularly useful for eliciting bNAb protective against HIV-1 infection. Prerequisite to testing this hypothesis is the biosynthesis of mHIVenv as non-infectious subunits of viral envelope proteins devoid of viral DNA/RNA, reverse transcriptase and p24, but retaining gp120 and gp41 in an immunoreactive form.

As a first step in the development of immunoprophylaxis potentially applicable to preventing HIV-1 transmission in human populations, we report here a process for biosynthesis and purification of plasma-derived HIV-1 (PHIV), an inactivation procedure that produced mHIVenv without chemical modification of the envelope proteins gp41 and gp120 that remained noncovalently bound, and shown to be non-infectious in vitro and in vivo.

2. Materials and methods

2.1. Materials

The HIV-1 employed for this study was one of the PHIV isolates from healthy blood donors with acute infection detected by HIV-1 RNA amplification test during the antibody-negative period [10]. Selection of PHIV as the starting material is supported by the fact that patients undergoing acute HIV-1 infection harbor in their plasma a single founder virus with CCR5-tropic phenotype and sensitive to in vitro neutralization [11–13]. Such PHIV can be difficult to grow in T-cell lines and require phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC) as a cell substrate [14]. Depleting CD8⁺ cells from the PBMC pooled from 3 to 4 donors provides an optimized cell substrate (OCS) for consistent expansion of different PHIV isolates [15].

2.2. Experimental procedures

The experimental approach for the biosynthesis of mHIVenv, devoid of p24, RT, and viral/host nucleic acids is illustrated in Fig. 1. It evolved from the emerging knowledge about the relative genetic homogeneity of HIV-1 isolates from the plasma of antibody-negative but acutely infected individuals [10,12,13], feasibility of producing large amounts of PHIV in OCS [15,16], purification of PHIV by removal of cellular microvesicles [17], inactivation of virions without chemical modification of the envelope proteins [18], and in vitro and in vivo testing for infectivity in SCID-hu Thy/Liv mouse model [19]. While we investigated four different PHIV isolates to determine their p24 antigen (NEK050A, Perkin–Elmer, Boston, MA) and gp120 antigen (Advanced Bioscience Laboratories, Kensington, MD) contents measured by enzyme immunoassays (EIA), for iteratively producing mHIVenv for experiments reported here we consistently worked with the stock PHIV₁₂₈₀ having 10⁵ TCID₅₀/mL. The HIV RNA copies/mL was measured by Amplicor HIV-1 Monitor Test, Version 1.5 (Roche Molecular Systems (Branchburg, NJ).

2.2.1. Virion expansion in optimized cell substrate (OCS)—The PHIV₁₂₈₀ stock isolate was iteratively expanded in OCS under BSL-3 conditions using disposable T-flasks. Typically, 50 × 10⁶ cells in 4 mL of RPMI were mixed with 1 mL stock virus and incubated at 37 °C for 2 h with intermittent mixing at 15 min intervals to maximize the acute infection of cells. The infected cells were washed once to remove free virions and resuspended in 25 mL R10 (RPMI1640, 10% fetal bovine serum, 40 IU/mL recombinant interleukin-2 [Proleukin, Chiron (now Novartis), Emeryville, CA], glutamine and penicillin-streptomycin), and incubated at 37 °C for 3 days. The cells were centrifuged and the supernatant was discarded because it had little to no detectable p24 antigen. The pelleted cells, mixed with 250 mL R10 containing 4.5 × 10⁸ fresh cells, were incubated at 37 °C, with half of the medium replaced at 3-day intervals and the cultures terminated on day 12. The cell-free supernatants centrifuged on days 6, 9, and 12 were stored at –80 °C. The pooled supernatants were thawed and passed through 0.45-micron filter to remove cell debris. Clarified supernatant (36 mL) was layered over 2 mL of 6% iodixanol (Opti-prep, Cosmo Bio USA, Carlsbad, CA) and then was centrifuged (20,000 rpm for 90 min at 4 °C) to pellet the virus. The virions pelleted from 2 L of supernatants were resuspended in 3 ml

phosphate-buffered saline (PBS, pH7.4). This virus preparation was used in the purification and inactivation steps as described below.

2.2.2. Virion purification—Virus preparations can contain immunogenic cellular debris that produce an aberrant antibody response to HLA antigens in immunized primates [20]. Since CD45 is not present on virions [17], we used anti-CD45 immunomagnetic beads (EasySep, Stemcell Technologies, Vancouver, BC-Canada) for immunoaffinity removal of CD45 + microvesicles and cell debris that would otherwise co-purify with the virions. A typical batch of 3 mL purified virions contained 4.7 µg/mL of p24 antigen.

2.2.3. Inactivation of purified virions—To inactivate the purified virions we used pharmaceutical-grade 2-hydroxypropyl-beta-cyclodextrin (BCD, Trappsol, CTD Inc, High Spring, FL), which is known to inactivate HIV-1 in a dose dependent manner and permeabilized the virions to release p24, RT, and viral RNA from inactivated virus particles that retained gp120 [18]. In a modification of the original method, we mixed 1 mL of purified virion suspension with 2 mL of 300 mM BCD in TNE buffer (0.01 M Tris-HCl, 0.1 M NaCl, 1.0 mM EDTA, pH 7.2), i.e. final 200 mM BCD, and incubated at 37 °C for 4 h to maximally dissociate p24 [21]. The mixture diluted to 19 mL with Tris-HCl buffer (50 mM Tris-HCl, 2 mM MgCl₂, pH 7.6) was filtered through a 100 kD Centricon Plus-20 device (Millipore Corp, Billerica, MA) to eliminate the p24, RT, viral RNA, and washed three times with Tris-HCl buffer. The inactivated virions recovered from Centricon filtration were suspended in 1 mL Tris-NaCl and reacted with 1000 units of protease-free Benzonase (BZ) at 37 °C for 24 h to maximally hydrolyze the residual viral/host DNA/RNA into 10–20 base oligonucleotides according to the manufacturer's product information data sheets (Novagen, Madison, WI). The gp120-containing mHIVenv was washed three times with PBS through a 30 kD Centricon Plus-20 device (Millipore Corp, Billerica, MA), and resuspended in 1 mL PBS. The final product was used as a prototype mHIVenv.

2.2.4. Analysis of mHIVenv proteins—The Centricon-separated 1.0 mL mHIVenv, with 21.7 ng/mL of gp120 was ultracentrifuged at 20,000 rpm for 90 min. After removing the supernatant, the “pellet” was resuspended in 100 µL of residual fluid and then examined by electron microscopy by Dr. Jun Liu at the University of Texas, Houston [9].

Virus-like particles carrying conformationally complex membrane proteins (‘Lipoparticles’) are a versatile tool for quantitative analyses of membrane protein interactions [22]. The 96-well microtiter plates, coated with Lipoparticles (LP) highly expressing dendritic cell-specific intracellular adhesion molecule 3 (ICAM-3)-grabbing non-integrin (DC-SIGN; CD 209) protein [23], were used in an experimental EIA for immunometric determination of gp120 and gp41 in mHIVenv (Integral Molecular, Philadelphia, PA). The prototype mHIVenv with 2.0 ng/ml of gp120 as determined by the standard EIA (Advanced Bioscience Laboratories, Kensington, MD) was serially diluted two-fold and 50 µL aliquots were added to the microtiter wells pre-coated with “ReadyReceptor” DC-SIGN LP. Control microtiter plates were coated with “Null” LP (without DC-SIGN). The microtiter plates were incubated for 45 min at 37 °C and washed thrice with PBS to remove any unbound proteins. The gp120 in mHIVenv, captured by DC-SIGN-specific antigenic site distinct from the CD4 receptor site [24], was probed with human monoclonal antibodies against gp120 (b12)

specific for the CD4-binding conformational antigen [25]. Another set of mHIVenv bound to DC-SIGN LP was probed with human monoclonal antibodies against gp41 (1240) [26–28]. After washing the plates thrice with PBS to remove the free monoclonal antibodies, murine anti-human antibodies were added and incubated for 30 min at 37 °C. Finally, after washing thrice the chemiluminescent HRP substrate was added to each well and chemiluminescence signal was quantified.

2.2.5. In vitro infectivity testing in co-cultures—Aliquots of untreated purified virions and mHIVenv were co-cultured with OCS for 12 days and the culture supernatant were tested for p24 antigen to assess HIV growth according to previously published protocols [15].

2.2.6. In vivo infectivity testing in SCID-hu Thy/Liv mouse model—The SCID-hu Thy/Liv mouse with human fetal thymus and liver tissue implanted in the capsule of the kidney is a valuable model for in vivo infectivity testing for HIV-1 in preclinical evaluation of antiviral efficacy [19]. Therefore, we employed it to test in parallel the infectivity of purified PHIV without any treatment and mHIVenv product derived from the inactivated virions. For this purpose 2 mL of purified PHIV containing ~5.0 ug of PHIV was freshly inactivated with BCD and BZ as described above and concentrated to 0.5 mL of mHIVenv. At the time of inoculation the mice are surgically opened up to directly inoculate 50 uL of the inoculum into the thymic tissue.

Four groups of mice, with four animals per group, were inoculated with serial 10-fold dilutions of the non-inactivated PHIV. A group of four mice were mock infected with culture medium as a control. Finally, a group of 10 mice from a second SCID-hu cohort was inoculated with 50 uL of the mHIVenv product. Thus a total of 30 mice were inoculated for in vivo infectivity studies in SCID-hu Thy/Liv mouse model. Six weeks post-inoculation the implants were tested for detectable p24 antigen (detection limit 5 pg/10⁶ cells). These experiments were carried out in compliance with all federal government guidelines and institutional policies.

3. Results

3.1. PHIV stocks

We proceeded with fresh aliquots of four different PHIV stock isolates (PHIV₁₂₈₀, PHIV₁₃₆₂, PHIV₁₃₇₂, and PHIV₁₃₈₆) of R5 phenotype and measured their levels of p24 and gp120 antigens by EIA. The results in Table 1 reveal the observed variation in p24 and gp120 levels and the corresponding variation in the p24:gp120 ratio. While gp120 levels were relatively constant among the cultures (2–3 ng/ml), 5-fold variation was seen in the p24 levels (74–388 ng/ml). As a result, 8-fold variation in the p24:gp120 ratio (25:1–200:1) was observed among the cultures containing various PHIV isolates. These results are not unexpected, given the variability associated with HIV replication in primary human lymphocytes [29].

3.2. Laboratory tests to monitor virus inactivation

The results of p24 and gp120 antigens as measured by EIA, and HIV RNA copies/mL as measured by Roche Amplicor assay in a batch of purified PHIV before and after inactivation with BCD and BZ are shown in Table 2. The purified PHIV contained 4718.1 ng/mL p24 and 23.6 ng/mL gp120, with 1.9×10^9 HIV RNA copies/mL. In contrast, the mHIVenv contained only 0.26% of the p24 without significant loss of gp120, and a dramatic decline to 80 HIV RNA copies/mL, a depletion of $7.4 \times \log_{10}$.

The in vitro infectivity titer of non-inactivated PHIV was 8618 TCID₅₀/mL in PHA-stimulated PBMC. In contrast, the aliquots of mHIVenv following inactivation with BCD and BZ, were completely negative in HIV co-culture with OCS. For comparison, we also tested an aliquot of the HIV-1 SF2 Vaccine stock stored in liquid nitrogen. The HIV-1 SF2, with in vitro titer of 10^5 TCID₅₀ [30], was determined to contain 6.4×10^9 HIV RNA copies/mL.

3.3. Electron microscopy (EM) of mHIVenv

Two different batches of mHIVenv examined for by negative staining in EM was reported show “amorphous material without any virus-like particles in the pellet” (data not illustrated). Subsequent EIA tests for gp120 performed on the ultracentrifuge pellet and the supernatant showed no significant difference. Presumably, the prolonged inactivation of purified virions by BCD reacted for 4 h and BZ reacted for 24 h appear to have disintegrated the virion particles into mHIVenv subunits.

3.4. Lipoparticle EIA: DC-SIGN-captured gp120 reveals noncovalently bound gp41 in HIVenv

The two-stage virus inactivation by BCD and nucleic acid hydrolysis by protease-free BZ was selected to prepare natural mHIVenv without any chemical modification of viral proteins that was functionally monitored by immunoreactivity of p24 and gp120 in standardized EIA (Table 2). Unlike the standardized kits for gp120, an EIA kit for gp41 is not available. The mHIVenv retained virtually all of gp120 after repeated filtration of the products through 100 kD and 30 kD Centricon Plus-20 devices as shown in Table 2. This result also suggested that viral inactivation procedures did not disrupt the natural trimeric gp41 into monomeric form filterable through the Centricon devices. Since EM revealed no virus particles, we investigated the possibility of emptied virions disintegrating into trimeric gp41 (123 kD) with noncovalently bound three molecules of gp120 to form mHIVenv protein subunits with an estimated molecular mass of ~500 kD. For this purpose we employed an EIA using microtiter plates coated with 150 nm Lip-oparticles with expression of DC-SIGN at levels 10–100 times greater than the native molecules in cells or membrane proteins (www.integralmolecular.com). Since DC-SIGN possesses tetrameric binding sites for gp120 distinct from the CD4-receptor site for infecting PBMCs [31], we reasoned that at low protein concentration most of the mHIVenv would bind to the DC-SIGN LP. The EIA results illustrated in Fig. 2 show that gp120 captured by DC-SIGN was reactive with monoclonal anti-gp120 (b12), and the non-covalently bound gp41 was similarly reactive with monoclonal anti-gp41 (1240). The modest reaction of mHIVenv with “Null” LP at a level lower than that with DC-SIGN is not surprising given that HIV can nonspecifically

bind to leukocyte and red cells membranes (ghosts) through non-CD4 and non-DC-SIGN binding sites [32].

3.5. In vivo infectivity testing of PHIV and mHIVenv

The results of in vivo infectivity testing of mHIVenv in the SCID-hu Thy/Liv mouse model are shown in Table 3. Note that all mice in Groups A, B, C, and D, inoculated with 10-fold dilutions of non-inactivated PHIV, were infected even at the lowest dose of 0.4 TCID₅₀. As expected, the mice in the control group E sham inoculated with culture medium were not infected. In contrast, when 50 uL of concentrated mHIVenv (4×) was inoculated into each implant of the group of 10 mice, none of the mice were infected (Group F). These results are consistent with in vitro infectivity results mentioned above (see Section 3.2). The in vitro infectivity testing by co-cultures in OCS and in vivo infectivity testing in SCID-hu Thy/Liv mouse model are complementary in establishing a dual safety of mHIVenv product. These results also demonstrate that the SCID-hu Thy/Liv mouse model is far superior to the in vivo infectivity testing in chimpanzees [30].

4. Discussion

4.1. PHIV is a key resource

The risk of HIV-1 infection harbored by antibody-negative blood donations was first recognized by co-culturing pooled PBMC and PCR testing [33], which formed a foundation for the evolution of donated blood screening by both antibody and nucleic acid testing (NAT) for HIV-1 infection, as well as hepatitis B and C. The plasma from blood donations testing positive for HIV RNA during anti-HIV negative period is exceedingly rare in the U.S. A. (1/1,847,429) [34]. The PHIV from such rare donations can only be isolated by co-culturing in human PBMC, and preferably in PHA-stimulated pooled PBMC as OCS [15]. The seminal discovery that sexually transmitted virus in acute HIV-1 infection (Fiebig Stage I/II) is a genetically homogeneous single virus of CCR5-tropic phenotype and neutralization-sensitive defined the possibility of PHIV for research and development of HIV vaccines capable of inducing bNAb [10–13,35]. It is conceivable that cloned PHIV expressed in established cell substrates will likely provide opportunities to produce new vaccine candidates for the prime-boost approach, which for the first time showed any efficacy after successive failures of all other reported clinical trials [36].

The levels of gp120 antigen in four different PHIV isolates were relatively uniform compared with the wide variations in p24 antigen levels (Table 1). This fact is reassuring for the development of natural mHIVenv as a pooled biological product representing different HIV clades.

4.2. Virologic safety

Large-scale co-cultures of PHIV with OCS in T-flasks require Biosafety level 3 (BSL-3) facilities. However, bioreactors offer a safe alternative for such co-cultures carried out under BSL-2 conditions, making it practical to grow large quantities of PHIV in OCS without availability of a BSL-3 facility [15,16]. Since HIV invariably establishes persistent infection, any risk of infection is unacceptable. Therefore, attenuated or inactivated HIV vaccine

candidates have not been meaningfully pursued in the past. This may change but for now such research is not in vogue.

The results of end point titration in OCS, and 0.4 TCID₅₀ infecting all inoculated SCID-hu Thy/Liv mice validate the dual safety testing in vitro and in vivo as more sensitive than the infectivity testing in chimpanzees [30]. A combination of prolonged inactivation with BCD for 4 h and BZ for 24 h not only thoroughly inactivated PHIV but appears to disintegrate the virion particles into subparticulate macromolecular mHIVenv proteins as subunits with an estimated molecular mass of ~500kD. The covalently bound trimeric gp41 in lipid bilayer should remain unaffected by cholesterol extraction with BCD and nucleic acid hydrolysis with protease-free Benzonase. The binding of gp120 to DC-SIGN Lipoparticles and detection of noncovalently bound gp41 opens the possibility of assembling 10–100 fold higher concentration of the mHIVenv on 150 nm virus-like particles than the 7–10 spikes of the HIVenv on native virions [9].

Further biophysical, and immunochemical characterization of mHIVenv is warranted to consider mHIVenv subunits as a better alternative than the monomeric gp120 used in the prime-boost protocol established in the large scale clinical trial carried out in Thailand [36]. Immunization of experimental animals is mandated by the recent report of gp41 subunit ‘viroosomes’ inducing mucosal antibodies protecting nonhuman primates against vaginal challenge with infectious virus [37]. The viral safety of mHIVenv reported here supports testing its immunogenicity and safety in baboons before possibly immunizing CCR5- 32 homozygous volunteers, who may receive pre-exposure prophylactic therapy [38]. Thus we foresee use of safe and effective mHIVenv to prepare env-specific immunoglobulin (env-HIVIG) for passive prophylaxis.

In summary, our preliminary results demonstrate the feasibility of biosynthesis of infection-free HIV-1 envelope proteins as a subunit for further research and development to open a new front in immunoprophylaxis against HIV infection.

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Fig. 1.

Procedures for deriving and testing natural non-infectious HIV envelope proteins. Shown are the major components of each of the three steps in this study: i) propagation of virus in primary cell culture, ii) inactivation of virus, and iii) testing of the final product for infectivity. Key features include the use of pooled PBMC after depleting CD8+ cells as optimized cell substrate for the production of PHIV, inactivation of virus using combination of beta-cyclodextrin and Benzonase, and *in vivo* safety testing in SCID-hu mice with human thymic and hepatic tissues implanted in their kidney capsule.

Fig. 2.

A) Diagrammatic representation of a mHIVenv subunit dissociated from inactivated PHIV (left) and a typical DC-SIGN Lipoparticle (right). Illustrated are three gp120 molecules, noncovalently assembled with membrane-bound trimeric gp41, forming a macromolecular mass of ~500 kD estimated without the remnants of virion lipids and matrix proteins shown as four spheres. The standardized 150 nm Lip-oparticles, expressing an estimated 100 molecules of DC-SIGN with putative tetrameric binding sites for gp120, are available from Integral Molecular in Philadelphia. B) and C) illustrate two graphs showing luminescence after reaction with monoclonal human antibodies to gp120 (mAb b12) and to gp41 (mAb

1240), respectively. The relative proportion of immunometrically detected gp120 and gp41 over a range of mHIVenv concentrations is reflected in the similarity of both plots.

Table 1

Levels of HIV p24 and gp120 in primary cell cultures that were infected with different PHIV isolates.

Virus isolate	P24 (ng/ml)	gp120 (ng/ml)	Ratio of p24/gp120
PHIV ₁₂₈₀	388.1	2.0	196.6
PHIV ₁₃₆₂	140.4	2.7	52.6
PHIV ₁₃₇₂	157.3	2.2	71.0
PHIV ₁₃₈₆	74.0	2.9	25.4

Table 2

Measurements of p24, gp120, and HIV RNA, and in vitro co-culture performed on non-inactivated PHIV and mHIVenv from PHIV inactivation by BCD and BZ.

Tests	Non-inactivated PHIV	mHIVenv	Depletion
HIV p24 ng/mL	4718.1	12.5	99.74%
HIV gp120 ng/mL	23.6	21.7	9.1%
HIV RNA copies/mL	1.9×10^9	80	$7.4 \times \log_{10}$
Co-cultures	Positive	Negative	100%

Table 3

Determination of in vivo infectivity of mHIVenv and serial 10× dilutions of non-inactivated PHIV inoculated in the implants of SCID-hu Thy/Liv mice.

Group	Mice (N)	TCID ₅₀ per implant	Number infected	p24 (pg/10 ⁶ cells)	Total cell yield (×10 ⁶)
A ^a	4	430	3/3	630 ± 93	150 ± 32
B ^b	4	43	3/3	750 ± 140	110 ± 38
C	4	4.3	4/4	460 ± 150	210 ± 45
D	4	0.43	4/4	470 ± 110	230 ± 84
E	4	Mock infected	0/4	Negative	680 ± 160
F	10	mHIVenv	0/10	Negative	90 ± 12

^aGroup A: Mouse #4 died (cause unknown).

^bGroup B: Mouse #6 no implant found at termination.