

Transmission of Retroviral Infection by Transfusion of Seronegative Blood in Nonhuman Primates

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Techniques such as polyclonal B cell activation with pokeweed mitogen (PWM) and polymerase chain reaction (PCR) analysis have documented the existence of simian immunodeficiency virus (SIV)- and human immunodeficiency virus type 1-seronegative but infected humans and nonhuman primates. To establish whether blood from such seronegative but PWM- and PCR-positive monkeys can transmit infection, naive macaques were transfused with whole blood ($n = 2$) or cultured cells and supernatant fluid ($n = 2$) from two seronegative but PWM- and PCR-positive sooty mangabeys. After transfusion, three of the four recipients seroconverted, and peripheral blood mononuclear cells from all four recipients secreted SIV-reactive antibodies upon polyclonal activation in vitro and were SIV-positive by PCR that used highly specific gag primer pairs and probe. In addition, CD8⁺ cells from all four recipients markedly inhibited replication of SIV in autologous cells in vitro. These data suggest caution in the sole use of serologic tests for the detection of retroviral infection and document the ability of such blood samples to transmit infection.

The transmission of human immunodeficiency virus type 1 (HIV-1) via contaminated blood transfusions has been well documented [1-5]. To prevent such transmission, comprehensive screening of blood donors, including serologic tests for the presence of anti-HIV-1 antibodies, has been very effective in the prevention of HIV-1 transmission by blood and blood products. Recently, however, several laboratories (including ours) have identified cohorts of individuals who have undetectable levels of serum antibodies against HIV-1 or human T cell leukemia virus (HTLV)-1 yet are infected with the respective viruses [6-9]. The tests that show HIV-1 or HTLV-1 infection in these seronegative individuals include detection of viral sequences by polymerase chain reaction (PCR) and in situ hybridization techniques and the finding that polyclonal activation of peripheral blood mononuclear cells (PBMC) in vitro leads to the secretion of readily detectable antibodies against HIV-1 [6]. Debate continues about the specificity and sensitivity of the assays, including problems associated with PCR analysis; however, the likely existence of a "window period" between infection and seroconversion could readily account for such findings. The window period varies and could last for months [9, 10].

The existence of seronegative but infected individuals is further strengthened by our studies of sooty mangabeys (*Cercocebus atys*), a nonhuman primate species housed at the Yerkes Regional Primate Research Center (YRPRC). This species is naturally infected with a simian immunodeficiency virus (SIV/SMM), and serologic tests indicate that ~15% of the adult animals (>5 years old) and ~40% of the rest (<5 years old) are seronegative for SIV/SMM [11].

Recently, we found that despite repeated negative serology (over months to years), PBMC from these seronegative mangabeys contain SIV-specific sequences (determined by PCR). In addition, SIV can be isolated from their blood, and polyclonal activation of PBMC in vitro leads to readily detectable antibodies specific for SIV *env* and *gag* proteins, similar to our findings of occult HIV-1 infection in a select group of humans at high risk for HIV-1 infection [6, 12]. Moreover, CD8⁺ cells from these seronegative mangabeys markedly suppress replication of SIV in autologous cells in vitro [13], a property previously shown to be exclusively present only in SIV- and HIV-infected nonhuman primates and humans, respectively [14, 15].

These findings of seronegative yet infected humans and nonhuman primates prompted us to determine whether blood transfusions from seronegative individuals can result in transmission of infection and disease. Here we report the data from an initial study.

Materials and Methods

Animals. Two naturally infected but seronegative sooty mangabeys that served as blood donors (FSg and FRf) were maintained in outdoor compounds that have adjacent indoor facilities; the pig-

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Instructions of the Committee on the Care and Use of Laboratory Animals were followed.

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tailed and rhesus macaques were housed in individual cages in indoor facilities. All animals were maintained at the YRPRC of Emory University. The animals used in this experiment and its design are described in table 1.

PBMC isolation and depletion of CD8⁺ cells. PBMC were isolated using a Percoll gradient. PBMC were incubated on ice with monoclonal mouse anti-human CD8 (Leu-2a) for 30 min. The cells were rinsed once, and then goat anti-mouse immunoglobulin, bound to magnetic beads (Dynabead, Long Island, NY), was added to the cells at a concentration of 10–50 beads/cell and incubated for 30 min on ice. The CD8⁺ cells were removed using a magnet (Dynabead). To recover the CD8⁺ cells, the beads were incubated overnight at 37°C in the presence of 10 units/ml interleukin-2 (IL-2).

Cell culture and pokeweed mitogen (PWM) stimulation. The cells were suspended at 2×10^6 cells/ml in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (all from GIBCO, Grand Island, NY). For polyclonal stimulation of B cells, PWM (GIBCO) was added at a final dilution of 1:200. Cells were cultured for 7 days in U-bottomed culture tubes or in 50-ml flasks at 37°C in a 7% CO₂ humidified atmosphere.

Preparation of donor blood, cultured cells, and supernatant fluid for transfusion. Heparinized blood from two repeatedly tested SIV-seronegative sooty mangabeys (FSg and FRf) was obtained, and within 1 h, 20 ml from FSg and 18 ml from FRf were transfused into prescreened uninfected SIV-seronegative pigtailed macaques 28H and F84315. These two monkeys were denoted recipients of whole blood transfusions.

For the transfusion of cultured cells and supernatant fluid, a total of 30×10^6 PBMC from the same two SIV-seronegative mangabeys were individually cultured at 2×10^6 cells/ml in media containing 0.2% phytohemagglutinin-P (PHA-P) for 5 days. The cells were then washed and cocultured with day 3 human PHA-P blasts in media containing 10 units/ml recombinant-IL2 (r-IL2; courtesy Hoffman LaRoche, Nutley, NJ) for 30 days. Supernatant fluid (SF) from individual cultures was collected every 3–4 days, and the cultures were

replenished with fresh media containing r-IL2. The SF was frozen at –70°C, and on the day of transfusion, all SF from each individual donor monkey was pooled and centrifuged at 150,000 g for 1 h at 4°C. The pellet from each was then added to autologous day 5 PHA-P blasts (1×10^7 cells), and the mixture of cells and pelleted virus from the supernatant fluid (CCSF) from FSg and FRf was transfused in a volume of 2.6 and 2.2 ml, respectively, into two prescreened (SIV-seronegative, PCR-negative, uninfected) recipient pigtailed macaques (130G and F84200).

Viral isolation from pigtailed macaques. PBMC from each recipient were cultured at 2×10^6 /ml in media containing 0.5% PWM for 7 days. The cells were then cocultured for 17–21 days in flasks with H-9 cells in media containing 10 units/ml r-IL2. Supernatant fluid from these cocultures was collected every 3–4 days for the measurement of reverse transcriptase activity (RT), as previously described [16], and cultures were replenished with fresh media. Controls were supernatant fluid from cultures of PBMC from an uninfected pigtailed macaque (negative control) and cultures from an experimentally infected rhesus macaque (positive control) done in parallel with cultures from the transfusion recipients. Each RT assay included solubilizing buffer alone, which provided background RT activity. The mean background RT activity was subtracted from the mean RT activity of each of the cultures. RT activity was considered positive only when the value obtained was ≥ 3 SD above that obtained with cultures from the uninfected control animals.

ELISA and Western blot assay. The ELISA for quantitation of SIV-specific antibodies was done as previously described [17], using SIV/SMM (Advanced Biotechnologies, Silver Spring, MD). Negative and positive controls for serum antibody levels were sera from uninfected and experimentally infected macaques, respectively. Negative and positive controls for detection of antibody in supernatant fluids of PBMC cultured with PWM were supernatant fluids of parallel cultures of PBMC from uninfected and experimentally infected macaques. The mean background (buffer alone) optical density (OD) value was subtracted from the mean OD of each experimental value.

The ELISA was considered positive only when the net mean OD value was at least 2 SD greater than the net mean OD obtained with corresponding sera and supernatant fluid from uninfected control animals. The SIV Western blot assay followed previously published protocol [18], using SIV/SMM as the antigen. Essentially, sera to be tested were diluted 1:50 in PBS (pH 7.4) whereas supernatant fluids of PBMC cultured with PWM were used undiluted. Supernatant fluids to be analyzed by Western blot were assayed on the day of harvest. Negative and positive sera and supernatant fluids were included with each Western blot and were the same samples used for the ELISA. Sera from seropositive control animals always gave at least six bands against the major proteins of SIV (*env*, *pol*, *gag*, etc.). Sera from uninfected macaques and seronegative mangabeys had no visible bands. The Western blot was considered positive only when at least two bands against the major *env*, *pol*, or *gag* proteins were visualized.

PCR. The primers and probe used in this study were chosen on the basis of highly conserved sequences of the SIV *gag* protein and corresponded to nucleotides 1568–1593 (sense strand), 2108–2084 (antisense strand), and 1678–1702 (probe) of the SIV_{smmb4} isolate [19]. This set of oligonucleotides has been shown to be cross-reactive and to detect *gag* sequences from various SIV and HIV-2 isolates but did not detect HIV-1 or HTLV-1 sequences. The PCR assay was done as previously described [12].

Table 1. Pigtailed macaques and seronegative sooty mangabeys studied.

Recipient nomenclature, date of birth	Weight* (kg)	Donor nomenclature	Recipients transfused intravenously with
28H, 4/1/88	2.56	FSg	WBT, 20 ml of heparinized blood
F84315, 10/9/84	7.54	FRf	WBT, 18 ml of heparinized blood
130G, 10/1/87	2.86	FSg	CCSF, 2.6 ml of in vitro cultured cells and supernatant fluid
F84200, 6/30/84	8.20	FRf	CCSF, 2.2 ml of in vitro cultured cells and supernatant fluid
2H, 2/1/88	2.56	—	Seronegative normal control
PCj, 8/18/84	14.08	—	Seronegative normal control

NOTE. WBT = whole blood transfusion, CCSF = cultured cells and supernatant fluid.

* Weight at time of transfusion.

Results

Serologic studies. Serum samples from the two whole blood transfusions and the two CCSF recipients obtained before and 1, 2, 4, 8, 12, and 16 weeks after transfusion were individually assayed for anti-SIV reactivity by ELISA. As seen in figure 1A, peak antibody reactivity was seen in serum samples 2 weeks after transfusion in one whole blood transfusion and one CCSF recipient. Whereas serum samples from the other whole blood transfusion recipient failed to show demonstrable SIV-specific serum antibody reactivity, a low but statistically significant titer ($P < .005$) was demonstrable in serum samples from the other CCSF recipient. A decline in SIV-specific antibody titers was seen in samples from each of the recipients after 4 weeks. Each assay included serum samples from two uninfected pigtailed macaques (negative controls) and two rhesus macaques experimentally infected with SIV/SMM (positive control).

Negative control values were always negative ($<1:10$), and the positive control values were always $>1:400$. Each of the serum samples was also tested for antibody reactivity, using an HIV-2 ELISA kit (Abbott, North Chicago), and the data obtained were essentially similar to those obtained using our laboratory SIV ELISA assay (data not shown). In addition, serum samples from each of the recipients 2 weeks after transfusion were analyzed qualitatively by Western blot assay. Sera from one whole blood transfusion recipient and two of the CCSF recipients showed reactivity against *gag* and *pol* proteins of SIV/SMM but not against all of the SIV proteins; however, sera from a rhesus macaque experimentally infected with

SIV/SMM reacted with more than six bands (data not shown). Peak antibody reactivity appeared to coincide with marked axillary and inguinal lymph node and spleen enlargement in the three recipients with high antibody titers but not in the whole blood transfusion recipient that lacked demonstrable anti-SIV reactivity.

In vitro polyclonal activation. Our laboratory has shown that polyclonal activation of PBMC in vitro leads to the synthesis and release of HIV-1- and SIV-reactive antibodies from infected but seronegative humans and nonhuman primates, respectively [6] (unpublished data). It was thus reasoned that such an assay might help in the detection of SIV exposure in the form of SIV-specific humoral immunity in these transfused monkeys. Thus, PBMC from each recipient at 1, 2, 4 and 20 weeks after transfusion (when sufficient cells were available) were also cultured with PWM for 7 days in vitro, and the supernatant fluid was collected and assayed for SIV antibody reactivity. As seen in figure 1B, while supernatant fluids from PBMC cultures of seronegative and uninfected control pigtailed macaques showed no detectable SIV antibody reactivity, supernatant fluids from all four recipients (whole blood transfused and CCSF, two each) showed significant SIV antibody reactivity as early as 1 week after transfusion. This was also true for animal 28H, which received whole blood and whose sera failed to show detectable levels of SIV antibody reactivity (figure 1A). Supernatant fluids from PBMC cultures of all macaques at 20 weeks after transfusion continued to show measurable SIV antibody reactivity although the corresponding sera was negative (figure 1B); thus,

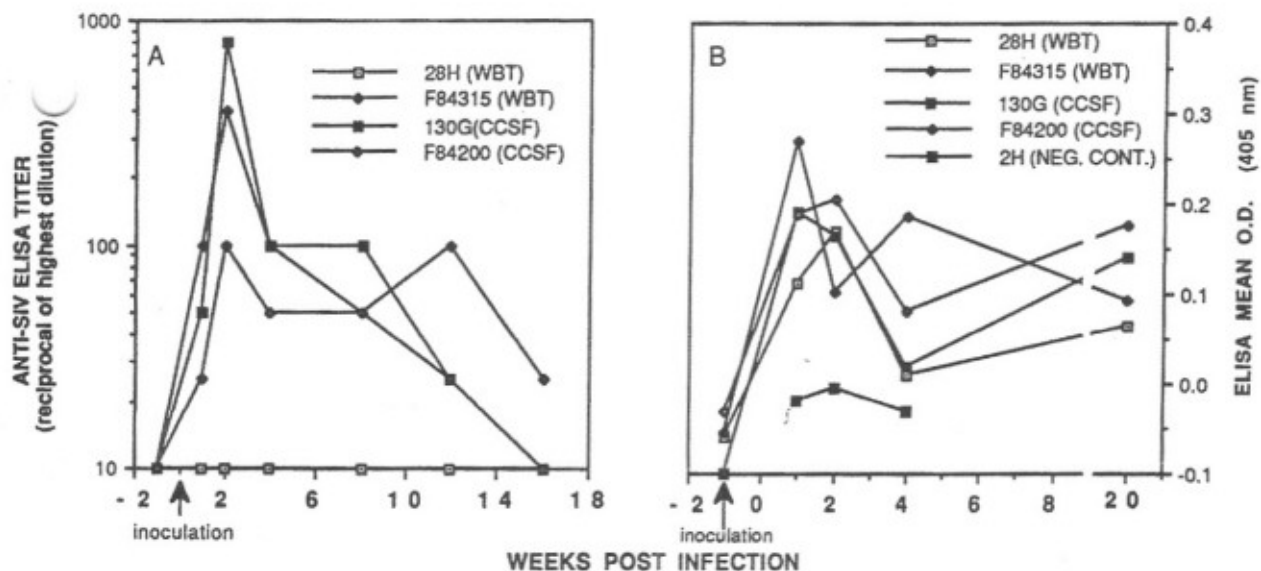


Figure 1. Detection of simian immunodeficiency virus (SIV)-specific antibodies as a function of time after transfusion. A, Serum titers of SIV-reactive antibodies. B, In vitro biosynthesis of SIV-reactive antibodies by pokeweed mitogen stimulation of peripheral blood mononuclear cells. Optical density (OD) readings (405 nm) reflect net values after subtraction of values obtained with media and uninfected rhesus control. All values represent mean of 3–4 wells; SD was never $>10\%$ of the mean. Positive controls (from experimentally infected seropositive rhesus macaque) gave OD readings of 0.4–0.6 (not shown). WBT = whole blood transfusion, CCSF = cultured cells and supernatant fluid.

seroconversion was followed by a seronegative period although the animals were infected (see below) and had circulating SIV antigen-primed B cells.

Presence of virus. PBMC from each transfused animal that were cultured with PWM were subsequently cocultured with H-9 cells. Table 2 shows that supernatant fluids from each of the four recipients showed low, yet positive, RT activity at each interval examined, whereas supernatant fluid from the two control uninfected pigtailed macaques (2H and PCj) lacked demonstrable RT activity. Positive controls were cocultures of PBMC from two rhesus macaques experimentally infected with SIV/SMM; data from one is shown in table 2 (data from the other was similar). Supernatant fluids from these positive control specimens gave values similar to those of supernatant fluids from the four transfusion recipients (table 2).

It has been shown that CD8⁺ cells from SIV- and HIV-1-infected monkeys and humans, respectively, markedly inhibit respective viral replication (as measured by RT activity) in vitro [14, 15, 20]. In an attempt to further augment our ability to detect the presence of SIV in the transfusion recipients, aliquots of PBMC from these animals obtained 8 weeks after transfusion were cultured in vitro with and without depletion of CD8⁺ cells. Similar cultures of PBMC from an uninfected pigtailed macaque served as negative control. As seen in table 3, supernatant fluids from all four transfusion recipients showed marked increases in RT activity in cultures depleted of CD8⁺ cells. Finally, supernatant fluids from cocultures of PBMC from each of the four transfusion recipients are now being collected and viral preparations pooled. These viral isolates are being analyzed for their in vitro SIV-specific growth characteristics and are being subjected to electron microscopic studies and nucleotide sequence analysis.

PCR analysis. PBMC from each of the four transfusion recipients were also analyzed 0, 4, 8, 12, and 16 weeks after transfusion for the presence of SIV by PCR. A set of gag primer pairs based on a conserved sequence present in all SIV isolates known so far [19] was used to amplify sequences in

DNA samples from the PBMC of each recipient, including negative (uninfected pigtailed macaque 2H) and positive controls (rhesus macaque 485 experimentally infected with SIV/SMM). As seen in figure 2, PBMC from all four transfusion recipients and the positive control contained sequences specific for SIV/SMM only after the transfusion. Such positive PCR results were seen in one whole blood transfusion and two CCSF recipients as early as 4 weeks after transfusion and at 16 weeks after transfusion in the other blood transfusion recipient (28H), which remains seronegative. While the signal detected in samples from 28H was relatively weak, it was repeatedly positive; no signal was given by samples from the negative controls. Repeated PCR analysis of PBMC samples at four different times after transfusion from each of these recipients (but not from negative controls) continues to show the presence of SIV-specific sequences up to 5 months after transfusion (latest samples analyzed).

Discussion

This study demonstrates that blood or blood products from otherwise seronegative but infected sooty mangabeys transfer infection into naive pigtailed macaques. Several issues are germane to these findings. The sensitivity and specificity of the ELISA and Western blot assays were rigorously scrutinized and tested by our laboratory. Most trivial issues, such as the detection of IgM versus IgG antibodies by the ELISA and the specificity of the protein band detected by Western blot were addressed by use of multiple isotype-specific antisera and synthetic peptides of SIV *env* and *gag* regions. These data clearly indicate that at appropriate intervals, sera and supernatant fluid from PWM cultures of the transfusion recipients contain antibodies against SIV.

It will be of great interest to learn if the pigtailed macaques given whole blood or CCSF will develop disease. This species has previously been shown to develop disease and die within 1 year of experimental infection with SIV/SMM-9. To

Table 2. Reverse transcriptase (RT) activity in supernatant fluids of cocultures of peripheral blood mononuclear cells (PBMC) from pigtailed macaque recipients of transfusions.

Animal, transfusion	Weeks after infection					
	1	2	4	8	12	16
28H, WBT	0*	6855	ND	29,712	15,637†	18,432
F84315, WBT	33,542	ND	16,430	16,605	26,187	11,172
130G, CCS	7717	18,037	15,125	29,142	58,422	14,466
F84200, CCSF	11,450	13,675	77,315	16,340	3500	24,957
2H, negative control	0	0	120	200	0	0
PCj, negative control	ND	ND	180	0	0	0
Rhesus, positive control‡	18,600	14,552	34,009	40,100	25,989	16,775

NOTE. PBMC were cultured with pokeweed mitogen (PWM) for 7 days and then cocultured with H-9 cells for 10–17 days. Supernatant fluids were collected at days 12, 15, and 17 and assayed for RT activity. Results are expressed as counts per minute (cpm)/ml and represent highest RT level obtained; RT activity in transfusion recipients was always >7500 cpm/ml except for one specimen from F84200 at week 12 after transfusion. WBT = whole blood transfusion, CCSF = cultured cells and supernatant fluid, ND = not done.

* 0 value represents raw cpm counts that were less than or equal to background levels.

† Supernatant fluid from day 32 of this coculture gave net 92,000 cpm/ml RT activity.

‡ Experimentally infected seropositive and asymptomatic rhesus macaque.

Table 3. Effect of CD8⁺ cell depletion on viral replication in pokeweed mitogen (PWM) cultures of peripheral blood mononuclear cells (PBMC) from pigtailed macaque transfusion recipients.

Animal	Transfusion	RT activity (cpm/ml)*	
		Unfractionated PBMC	CD8 depleted PBMC
28H	WBT	3250 (-)	18,687 (+)
F84315	WBT	3820 (-)	13,152 (+)
130G	CCSF	6092 (+)	12,295 (+)
F84200	CCSF	2977 (-)	9507 (+)
2H	-	2798 (-)	1971 (-)

NOTE. Unfractionated or CD8-depleted PBMC from each transfusion recipient at 8 weeks after transfusion and from an uninfected control pigtailed macaque were incubated *in vitro* with PWM for 10 days, and the supernatant fluid was collected and assayed for reverse transcriptase (RT) activity. No coculture was done. WBT = whole blood transfusion. CCSF = cultured cells and supernatant fluid.

* RT values were positive (+) or insignificant (-).

date (6 months after transfusion), all four recipients have no signs of disease except for transient inguinal and axillary lymph node and spleen enlargement. If these pigtailed macaques develop disease and die, the data will suggest that SIV from sooty mangabeys can transmit infection and also disease. However, it could be argued that over time the pigtailed macaques may selectively allow the growth of a variant (similar to SIV/SMM-9) that may induce disease. If such is the case, the PCR analysis of PBMC and subsequent sequence analysis of the isolates from the diseased macaques should help establish the selective propagation of such SIV variants. On the other hand, if the pigtailed macaques remain infected but neither develop disease nor die, the data would strongly argue in favor of the concept that an SIV variant that selectively propagates in seronegative mangabeys is not only distinct but also not a disease-inducing variant. Furthermore, such data would help establish that SIV/SMM-9 is a tissue culture-propagated variant of SIV that was selected for its unique growth and disease-inducing characteristics. The transfusion recipients could also theoretically clear the virus completely. Although very difficult to prove, negative data such as the inability to isolate virus *in vitro* even after depletion of the CD8⁺ cells and failure to detect viral sequences by PCR could indicate viral clearance.

Only one of the whole blood transfusion recipients seroconverted; this may be related to viral dose. Data from PCR and viral RT activity plus our ability to culture virus clearly show this macaque is infected. Follow-up studies will show if this recipient seroconverts or develops disease.

All three of the other recipients, after transient seroconversion, have reverted to a seronegative state. All three remain infected, but their serum lacks detectable SIV reactive antibodies. The precise reasons are not known; possibly humoral response against SIV proteins is potentially regulated by CD8⁺ T cells, and induction of such regulatory T cells follows the initial antibody response. This could explain our abil-



Figure 2. Results of polymerase chain reaction analysis of DNA samples from peripheral blood mononuclear cells of two pigtailed macaques (28H, F84315) that received whole blood and from two (130G, F84200) that received cells and supernatant fluid from seronegative sooty mangabeys. Lane A, rhesus macaque 485 experimentally infected with SIV/SMM (positive control); lane B, uninfected pigtailed macaque (2H, negative control); lane C, seronegative sooty mangabeys (FRf) that served as donor of blood for transfusions; lanes D-G, DNA samples from F84315, 28H, 130G, and F84200 before transfusion; lanes H, J, K, DNA samples from F84315, 130G, and F84200 4 weeks after transfusion; lane I, DNA samples from 28H 16 weeks after transfusion.

ity to detect SIV-primed B cells by polyclonal activation *in vitro*. Depletion of CD8⁺ T cells before *in vitro* culture leads to enhanced secretion of the SIV- and HIV-1-reactive antibodies, as previously shown by our laboratory [6, 21]. It is also possible that this seronegative state is related to dose of virus or its variant and that after initial immune response, the virus becomes and remains latent for an unknown period. Possibly the host immune response keeps the seronegativity in check until and unless a further immune insult triggers viral replication and subsequent disease. In addition, cofactors may play a role in the development of HIV-1-induced disease [22]. Routine follow-up of the macaques transfused in our study may help determine the answers to this perplexing puzzle.

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