Pig-tailed Macaques Infected with Human Immunodeficiency Virus (HIV) Type 2$_{GB122}$ or Simian/HIV$_{89.6p}$ Express Virus in Semen during Primary Infection: New Model for Genital Tract Shedding and Transmission

Jennifer K. Pullium,1,4 Debra R. Adams,1 Eddie Jackson,2 Caryn N. Kim,1 Dawn K. Smith,3 Robert Janssen,1 Kenneth Gould,3 Thomas M. Folks,1 Sal Butera,1 and Ron A. Otten1

Characterizing human immunodeficiency virus (HIV) expression in semen during primary infection remains essential to understanding the risk of sexual transmission. This investigation represents the first systematic evaluation of male genital tract shedding to use a nonhuman primate model, including the impact of exposure route and viral virulence. Male macaques were inoculated with either a chronic disease-causing virus (HIV-2$_{GB122}$; n = 4 intravenous; n = 4 intrarectal) or an acutely pathogenic simian/HIV strain (SHIV$_{89.6p}$; n = 2 intravenous). All macaques were systemically infected, and seminal plasma virus-associated RNA (vRNA) levels were ~10-fold lower than those in blood. In HIV-2$_{GB122}$ infection, seminal virus was delayed by 1–2 weeks compared with that in blood. Intrarectal inoculation resulted in a shorter duration of seminal vRNA expression and intermittent seminal cell provirus. No delays, higher peaks (~50-fold), or longer durations in seminal virus expression were noted for SHIV$_{89.6p}$ infection. This novel model definitively establishes that virus dissemination results in early peak seminal levels and provides a basis for evaluating interventions targeting male genital tract expression.

Most new human immunodeficiency virus (HIV) infections are acquired via sexual transmission involving seminal fluids [1, 2]. Analysis of human specimens has demonstrated the presence of both cell-associated and cell-free virus in semen [3, 4]. Sophisticated fractionation experiments have provided solid evidence that T lymphocytes and macrophages in semen can harbor virus. There was, however, no convincing virus association with spermatozoa [5]. At present, the relative contributions of cell-associated versus cell-free virus in semen for successful transmission remain undefined.

In general, HIV-1 levels in human semen have been found to be lower and much more intermittent than are those in peripheral blood [3, 6, 7]. Identification of differing viral populations expressed in semen versus blood has indicated that the male genital tract may serve as a distinct compartment for HIV-1 replication [6, 8–13]. Controversy exists amid disparate findings regarding the impact of blood virus levels, peripheral CD4 cell counts, and disease stage on seminal virus shedding [7, 11, 14]. The existing human data collectively indicate that HIV-1 shedding in the male genital tract can occur at any time during the course of infection [reviewed in 2].

Recent human studies [15–17] have assessed the impact of antiretroviral therapy on seminal HIV-1 burden during chronic infection, in an effort to alter overall transmissibility. Evaluating virus replication during early primary infection, especially in the genital tract compartment, however, remains essential to understanding sexual transmission potentials that may be perpetuating the pandemic. Very limited human data suggest that virus expression occurs in semen during primary infection [18–21], which may correlate with the greatest period of trans-
mission risk [21]. Comprehensive characterization of the earliest virologic events after exposure requires appropriate animal models, because problems with routine recognition of acute retroviral syndromes preclude such studies in humans.

The only previous attempt to investigate male genital tract shedding in nonhuman primates [22] was hampered by insensitive culturing methods, as were early human investigations. The main hurdle in using other macaque systems (rhesus) to evaluate seminal shedding has historically involved specimen coagulation problems immediately following collection, as highlighted elsewhere [17]. In the present study, we have overcome these logistical difficulties, and we report the first systematic evaluation of male genital tract virus shedding in a nonhuman primate model for acute-phase HIV infection. Important aspects, not feasible to investigate during human HIV infection, were explored in this study, such as the timing, level, and duration of seminal virus expression early after exposure; the impact of exposure route on virus-shedding characteristics; and the effect of virus virulence on expression from the male genital tract.

Methods

Animals and housing. Ten male pig-tailed macaques (Macaca nemestrina), 4–9 years of age and weighing 6.0–12.0 kg (Charles River Laboratories), were used in this study. These animals were housed individually under biosafety level 3 containment conditions. The cohort tested negative for malaria, tuberculosis, simian immunodeficiency virus (SIV), simian retrovirus type D, HIV-1, and HIV-2 at baseline.

Virus stocks and inoculations. The utility of HIV-2GB122, a primary isolate derived from an AIDS patient residing in Bissau, Guinea Bissau (West Africa), for productively infecting nonhuman primates has been documented elsewhere [23–25]. A cell-free virus inoculum was generated by limited propagation of the original human isolate onto a mixture of phytohemagglutinin-p (PHA-p)-activated peripheral blood mononuclear cells (PBMC) from 4 source macaques. The titer of the cell-free virus challenge stock was determined by standard coculturing techniques [26, 27] and consisted of 100 TCID per milliliter. Intravenous (iv) exposures consisted of 1 mL of virus stock injected into the saphenous vein. Intrarectal (ir) virus exposures involved atraumatic inoculation of cell-free virus (5 mL per exposure) into the rectum via a sterile gastric feeding tube. The ir inoculations were separated by 30 min, during which the anesthetized macaques remained recumbent to promote localized absorption. Specific strain characteristics of simian/HIV, SHIV89.6p, have been detailed elsewhere [28, 29]. The virus stock used for this investigation was obtained from a recent virus expansion on rhesus macaque PBMC (F. Villinger, personal communication) that was passaged onto PHA-p-blasted pig-tailed macaque PBMC with a starting MOI of 0.01. SHIV89.6p (derived from pig-tailed macaque PBMC) was propagated for 28 days and yielded a final stock with sufficient titer (100 TCID/mL) to be used for in vivo inoculations; iv exposures consisted of 1 mL of virus stock injected into the saphenous vein.

Specimen collection and processing. Longitudinal blood specimens were collected and were processed to isolate PBMC and plasma, cryopreserve PBMC, and derive cellular lysates, as described elsewhere [24]. Whole-blood and serum specimens were also routinely collected for blood chemistry analysis and complete blood cell counts. Semen specimens were obtained by mild rectal probe electrostimulation (RPE), which produces rhythmic contractions of pelvic muscles, resulting in emission of semen, usually yielding lower sperm concentrations compared with a true ejaculation [30]. A rectal probe (2.0–2.5 cm in diameter) with 2 flush-mounted electrodes was inserted into the rectum of anesthetized macaques. Mild electrostimulation required for emission of semen ranged between 4 and 15 V. Current was delivered intermittently, in a sequence of ~3 s of stimulus and 3 s of rest for a maximum of 15 min. The current was progressively increased until an emission resulted. After collection, semen was incubated at 37°C for 30 min, to allow the semen to liquefy as much as possible. Semen was then diluted 1:1 with RPMI containing 1000 μg/mL streptomycin and 1000 U/mL penicillin and was centrifuged at 600 g for 15 min [31]. The seminal plasma supernatant was recovered for cell-free virus detection and quantification. Seminal cell pellets were lysed and were evaluated for the presence of cell-associated provirus. Evidence derived from human specimens indicated that cell-associated virus localizes exclusively to mononuclear cells present in semen [5]. Thus, no attempts were made to minimize the amount of spermatinzoa in seminal cell specimens harvested for analysis.

Cell-associated provirus detection. Nested DNA–polymerase chain reaction (PCR) techniques were used to monitor the establishment of HIV-2env or SHIV89.6p provirus in PBMC and seminal mononuclear cells. The sensitivity (≥10 copies/10^6 cells) and high specificity of HIV-2 proviral DNA-PCR assays with a 10^6 PBMC input have been described elsewhere [24]. Seminal cell extracts were derived for nucleic acid purification by using a standard Nuclisens protocol (Organon Teknika). Because routine seminal cell counts were not feasible for each collection, owing to the potential for substantial sample loss, primary amplification of β-actin was used to ensure that cellular input amounts were standardized before subsequent virus detection. Duplicate input samples, corresponding to ~20% of the total specimen obtained, were used for virus-specific nested PCR analysis, with a maximum input of 4 × 10^6 mononuclear cells. All nested PCR reactions were performed as described elsewhere for PBMC specimens [24]. The presence of amplifiable DNA in both PBMC and seminal cells was confirmed by β-actin–specific PCR by use of the primers BAF1 (5'-GTGCTATCC-CGTGACGCCTCT-3') and BAR1 (5'-GGCCGTGGTGGTGAA-GCTGTA-3'). The primers were derived from the human β-actin mRNA sequence (GenBank accession number X00351). Cycler conditions were identical to those used for HIV-1 protease PCR [24]. SHIV89.6p provirus detection was accomplished in a similar manner, except that HIV-1 env-gp41 was the targeted region for nested DNA-PCR amplification using highly conserved and sensitive primer sets [32].

Cell-free virion-associated RNA (vRNA) quantitation. A reverse transcriptase–mediated PCR (RT-PCR) prototype assay system, similar in design to that originally detailed by Kwok and colleagues [33], was used for quantitative measurement of vRNA in blood and seminal plasma. Specific details regarding genomic positions of the utilized long terminal repeat (LTR) primers and assay conditions have recently been reported for monitoring HIV-
2 vRNA in the context of dual infections with HIV-1 [34]. Very few base-pair mismatching among this LTR primer set for SIV strains has enabled this vRNA quantitative assay to monitor these viruses effectively, as well as SHIV89.6p, as documented recently elsewhere [35]. Deviation from assay methodology reported earlier related to the desire to standardize extraction procedures for blood and genital tract specimens, to avoid any possible PCR inhibition [3]. Toward this end, vRNA from 150 μL of blood or seminal plasma, along with a spiked RNA quantitation standard (25 copies), was extracted by using Nuclisens methodologies (Organon Teknika). Single-tube RT-PCRs (rTth DNA polymerase, Roche Molecular Systems HIV-1/HIV-2). Reactive samples were confirmed by HIV-1/HIV-2. Sensitive detection of virus-specific serologic responses was achieved by using a synthetic peptide EIA (Genetic Systems HIV-1/HIV-2). Virologic characterization after HIV-2GB122 infection. Macaques infected with HIV-2GB122 were DNA-PCR positive for provirus in PBMC by week 1 after inoculation and at all time points through 12 weeks (table 1). Furthermore, all 8 macaques had ≥1 seminal cell provirus–positive result during the course of study; however, detection of virus signals in genital tract secretions was much more intermittent in comparison with that in peripheral blood, especially in the ir group (table 1). The last positive signal detected in the ir group was at week 4 after inoculation, whereas multiple seminal cell provirus–positive signals were observed for the majority of those in the iv group, with week 8 representing the latest detection time point. During 12 weeks of monitoring, 13 (46%) of 28 seminal cell specimens collected from the iv group were provirus positive, versus 5 (18%) of 28 specimens collected from the ir group (P = .0437, Fisher’s exact test).

The onset of detectable blood plasma virus loads for all 8 HIV-2GB122–infected macaques (figure 1A) occurred at week 1. Significant differences (P = .0262, unpaired t test) were observed between the iv and ir groups at this very early time point (mean values of 1.0104 vs. 1.0143 copies/mL, respectively), which may reflect slower virus dissemination due to ir inoculation. Peak vRNA levels were reached by weeks 1–2 after inoculation in the iv group (mean, 1.0164 copies/mL; range, 1.0105–1.0167 copies/mL) and by weeks 2–3 after inoculation in the ir group (mean, 1.0192 copies/mL; range, 1.0163–1.0213 copies/mL). No significant differences in absolute levels achieved were noted (P = .0842, unpaired t test), and vRNA expression for the majority of macaques in both the iv and ir groups remained above the quantitation limit at week 6 after inoculation (figure 1A).

Seminal plasma vRNA levels (figure 1B), compared with levels in blood plasma, were delayed by ≥1 week in all but 1 macaque (iv group); were quantitatively lower (mean peak, 1.0149 copies/mL; range, 1.0241–1.0663 copies/mL for the iv group, and mean peak, 1.0206 copies/mL; range, 1.0173–1.0335 copies/mL for the ir group); and were much more transient in nature with regard to detectability, especially in the ir group. It is interesting that the

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NOTE. A plus sign (+) represents successful nested DNA–polymerase chain reaction (PCR) amplification and detection of conserved pol–protease gene sequences for HIV-2GB122–exposed macaques or conserved env–gp41 sequences in the case of SHIV89.6p-exposed macaques. A minus sign (−) indicates that no virus-specific signal was detected, whereas the external cellular (β-actin) DNA-PCR control reaction was positive. ir, Intrarectal; iv, intravenous; NT, not tested.

### Results

To study the dynamics of virus load in both blood and semen during acute HIV infection, 10 male macaques were stratified into groups receiving a single iv dose corresponding to 100 TCID of HIV-2GB122 (n = 4); 2 ir doses (1000 TCID total) of HIV-2GB122 (n = 4); or a single iv dose (100 TCID) of SHIV89.6p (n = 2). Multiple inoculation routes were utilized to determine the impact of exposure route on genital tract shedding. Acutely pathogenic SHIV89.6p was compared with the more chronic HIV-GB122, to determine any role of viral virulence on genital tract shedding. All inoculations outlined in this investigation resulted in systemic infection.
Figure 1. Longitudinal virion-associated RNA (vRNA) levels in blood plasma (A) and seminal plasma (B) through 12 weeks after intravenous (n = 4; solid symbols) or intrarectal (n = 4; open symbols and dashed lines) exposure to human immunodeficiency virus type 2 (HIV-2GB122). Longitudinal vRNA levels in blood plasma (C) and seminal plasma (D) through 12 weeks after intravenous (n = 2) exposure to simian/HIV (SHIV89.6p). Results are reported as log_{10} copies/mL. The sensitivity limits are 100 copies/mL (as indicated on each graph), with a 50 µL sample equivalent input into the assay system.

Delay in onset of detectable seminal plasma vRNA did not translate into a significant delay in the timing to achieve peak levels, compared with that observed for blood plasma (P = .0824, unpaired t test). The trend toward higher peak levels in the iv versus the ir group (0.53 log_{10} difference among mean values) was not significant (P = .0821, unpaired t test). By week 6 after inoculation, seminal plasma vRNA levels for these macaques fell below the limit of quantitation, with no rebound through week 12.

Virologic characterization after SHIV89.6p infection. By comparison, SHIV89.6p infection also resulted in early (week 1) detectable provirus in PBMC (table 1), although continuous detection was observed for only 1 macaque (2FW). It is noteworthy that provirus-positive seminal cells were detected earlier, at week 1 after inoculation, in both SHIV89.6p-infected macaques, compared with those in the HIV-2GB122 group (table 1). The number of positive genital tract specimens (9 of 12) was also greater over the 12-week study course, although it did not reach significance (P = .1654, Fisher’s exact test).

Initial detection and peak vRNA levels (range, 10^{6.2}–10^{6.5} copies/mL) in blood plasma for both SHIV89.6p-infected macaques occurred at week 1 after inoculation and remained above the detection limit at week 12 (figure 1C). The mean peak vRNA value observed for these macaques (10^{6.37} copies/mL) was 1.73 log_{10} higher than that for the HIV-2GB122-infected iv group (10^{4.64} copies/mL). Seminal plasma vRNA levels (figure 1D) in these macaques also had higher peak levels (range, 10^{4.5}–10^{5.5} copies/mL) at weeks 1–2 after inoculation but remained lower than those noted for corresponding blood plasma.

Seminal plasma expression declined by weeks 3–4 after inoculation, reaching a magnitude similar to that observed for HIV-2GB122-infected macaques. At weeks 6 and 8 after inoculation, vRNA levels for 1 macaque (2FW) fell below quantitation limits; however, both SHIV89.6p-infected macaques were found to shed genital tract virus again at week 12 (figure 1D).

A single SHIV89.6p-infected macaque (3DK) became ill at week 15 after inoculation, with evidence of a ruptured spleen, and was promptly euthanized. Histopathologic analysis revealed splenic lymphoid hypoplasia similar to that found in previous studies [36]. The testes showed atrophy of the seminiferous tubules and hypospermatogenesis, which is consistent with the findings of postmortem studies in humans [37]. This is the first report of such findings in SHIV89.6p-infected macaques. All major organs, including testes, from 2 age-matched HIV-2GB122-infected macaques that were euthanized and un-
derwent necropsy at week 12 after inoculation were histologically normal. Further study involving additional macaques and a greater length of follow-up would be required to document other evidence of localized or systemic pathogenic consequences of infection.

_Virus-specific antibody (Ab) responses and lymphocyte fluctuations during primary infection._ As anticipated, all 8 HIV-2<sub>GB122</sub>-infected macaques developed EIA-reactive, Western blot (WB)-confirmed Ab responses by week 3 or 4 after inoculation (titer range, 10–25,600). The strength, timing, and duration of the systemic Ab responses observed were consistent with those we have observed previously in this macaque model for iv or vaginal route exposures [23, 25]. Virus-specific, WB-confirmed Ab responses were also observed in both SHIV<sub>89.6p</sub>-infected macaques, starting at 6 or 8 weeks after inoculation. These responses, however, were much lower (titer range, 10–200) than were those of the HIV-2<sub>GB122</sub>-infected macaques.

No overall differences were observed in the CD<sup>4+</sup> (figure 2A) or CD<sup>8+</sup> (figure 2B) cell populations among the HIV-2<sub>GB122</sub>-infected macaque groups. Notable CD<sup>4+</sup> cell losses, accompanied by increased CD<sup>8+</sup> cells, were observed for most macaques early within primary infection (2–4 weeks after inoculation), with distinct rebound patterns characteristic of HIV-2<sub>GB122</sub> replication in pig-tailed macaques [23]. SHIV<sub>89.6p</sub> infection resulted in a rapid total CD<sup>4+</sup> cell depletion by week 2 after inoculation that was maintained for the remainder of the study course (figure 2C). This severe depletion is a hallmark of infection for acutely pathogenic SHIV strains. Corresponding increases in CD<sup>8+</sup> cells (figure 2D) were also observed.

### Discussion

We sought to model male genital tract shedding in nonhuman primates (pig-tailed macaques) during primary retroviral infection. Novel aspects reported here include (1) longitudinal collection and virologic evaluation of macaque semen specimens after exposure; (2) direct assessment of cell-free and cell-associated virus within macaque seminal fluid versus peripheral blood; (3) the impact of distinct exposure routes (iv and ir) on seminal virus shedding, using the chronic HIV-2<sub>GB122</sub> strain; and

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**Figure 2.** CD<sup>4+</sup> and CD<sup>8+</sup> cell population profiles for human immunodeficiency virus type 2 (HIV-2<sub>GB122</sub>)-inoculated macaques (A and B) and for simian/HIV (SHIV<sub>89.6p</sub>)-inoculated macaques (C and D). Independent cellular baseline values for each animal were calculated from blood specimens collected before virus inoculation and were set at 100% for week 0. Results are presented as percentages of established baseline values, to detect fluctuations over the course of the 12 weeks after virus exposure.
(4) seminal and peripheral virus expression after infection with a chronic versus a highly pathogenic virus. Furthermore, this report documents the first attempts at infecting pig-tailed macaques with the highly virulent virus, SHIV

A majority of earlier human genital tract investigations were limited to chronically infected subjects [6, 38], whereas recent efforts have been more successful in identifying limited numbers of individuals early after HIV-1 acquisition [18, 19, 39]. Despite the successes of these human studies, an animal modeling system remains crucial to understanding the very earliest events occurring after infection. In the only previous report, which was limited because of insensitive culturing methods, virus-infected mononuclear cells were detected in just 2 of 10 semen specimens collected from a single chronically SIV-infected rhesus macaque [22]. In contrast, we used molecular methods for retroviral detection, which have been shown to have enhanced sensitivity in human studies [3, 38, 40, 41].

Elegant human studies have shown that virus levels observed in human semen were lower than those in peripheral blood and were more intermittent, probably because of local environmental (e.g., possibly mucosal immune-based) pressures and compartmentalization or selected growth of virus quasi species [3, 6, 7, 42]. These trends were also evident in this animal model study. In particular, for HIV-2

signals were also noted during both HIV-2GB122 and SHIV89.6p infection. In contrast, SHIV89.6p infection of pig-tailed macaques resulted in high levels of virus replication in both peripheral and genital tract compartments, with no delays in the appearance of cell-free or cell-associated virus in seminal fluid. Rapid CD4+ cell depletion and extremely high blood plasma vRNA levels have been the hallmarks of this acutely pathogenic virus in the rhesus system [29, 44], characteristics that have been extended to pig-tailed macaques by our report. Comparing pig-tailed macaque infection with SHIV

infection, coupled with seminal cell provirus detection, confirm limited existing human data [18–20, 43] showing that the male genital tract serves as an early site of dissemination and productive infection after retroviral exposure.

Furthermore, our investigation provides the first indication of possible differences in the longevity of seminal virus shedding as a function of exposure route. Overall, HIV-2

infection of pig-tailed macaques by our report. Comparing pig-tailed macaque infection with SHIV

versus HIV-2

infection. Also, the lack of seminal plasma virus at weeks 6 and 8 after inoculation that was noted for one of the 2 SHIV

infected macaques highlights the transient nature of genital tract virus expression, even in light of the pronounced virulence of the infecting strain used in this study. It is also interesting that the increased viral virulence observed in both macaques resulted not only in a more prolonged shedding in semen, but also in a longer seronegative window. By extrapolation, such observations are of great concern in humans, in whom infection with a highly virulent HIV strain may be missed by standard serologic detection methods, most likely increasing transmission potentials during this period of high infectiousness.

A potential weakness of this investigation, as with most other nonhuman primate modeling studies, was the ability to detect only marked differences among measured parameters because of the small number of animals per experimental group. Also, collection of semen by RPE may not be entirely representative of a true ejaculate, although detection and characterization of both cell-free and cell-associated virus shedding during primary infection were successful. Furthermore, extrapolation of results from any animal model to the human condition remains a constant challenge. The virus-host interactions that result from HIV-2

or SHIV

infections seemingly represent minority
extremes in virus virulence and resulting pathogenesis, compared with most human infections.

Assessing early genital tract events remains vital to understanding factors associated with sexual transmission of HIV. The ability to address these critical issues in the pig-tailed macaque system, with males or females [25], by using chronic or acutely pathogenic viruses enables important transmission questions to be more easily investigated. Transmission-related parameters, such as virus threshold levels and the relative contributions of seminal plasma versus seminal cell virus required for successful in vivo transmission, can now be better explored by using semen specimens collected from infected pig-tailed macaques.

In summary, this report provides vital insight into early events associated with HIV expression in the male genital tract and establishes a reproducible nonhuman primate system in which to study factors that govern transmission risk among infected individuals.

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References


