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Systemic Infection and Limited Replication of SHIV Vaccine Virus in Brains of Macaques Inoculated Intracerebrally with Infectious Viral DNA

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SHIV deleted in two accessory genes, Δ*vpu*Δ*nef* $SHIV_{PPC}$, functioned well as a vaccine against later challenge with highly pathogenic $SHIV_{KU}$, and it was able to reach the brain after oral inoculation of live virus. In this study, the proviral genome cloned into a plasmid was inoculated as DNA intracerebrally and spread systemically. Few regions of the brain had detectable proviral DNA by real-time PCR. Two measures of virus replication, detection of viral mRNA expression and circular proviral DNA, were negative for those brain regions, with the exception of the infection site in the right parietal lobe, whereas lymphoid tissues were positive by both measures. Histopathological analyses of all the sampled brain and spinal cord regions did not reveal any abnormalities. Despite intracerebral inoculation of the viral DNA, the brain was not targeted for high levels of virus replication.

INTRODUCTION

Live SHIV vaccines, created by deletion of regulatory genes *vpu* and *nef,* have been used in macaques in our laboratory to test proof-of-concept ideas regarding safety of the agents following oral/systemic routes of inoculation, and definition of the nature of protection against replication of pathogenic viruses given as challenge (Lamb-Wharton *et al.,* 1997; Joag *et al.,* 1998; Kumar *et al.,* 2001). In earlier reports, we had shown that ΔvpuSHIV$_{PPC}$ (Joag *et al.,* 1998), whose genome was derived originally by serial passage of SHIV-4 (Li *et al.,* 1992), a chimera of HIV-1_{HXBc2}, and SIV_{mac}239 in macaques, had proven to be safe and highly efficacious after a single oral inoculation of the agent into macaques (Joag *et al.,* 1998; Silverstein *et al.,* 2000). The virus had undergone productive replication in lymphoid tissues for about 6 weeks, after which it could be isolated only rarely from lymph node cells, even though its DNA persisted for long periods in these cells (Silverstein *et al.,* 2000; M. Smith *et al.,* unpublished observations). We then deleted *nef* from the genome of this virus, creating Δ*vpu*Δ*nef* $SHIV_{PPC}$, and examined the biological properties of this agent in six macaques that were inoculated orally with the agent (A. Kumar *et al.,* unpublished observations) and in four others into which the agent had been passaged by serial transfer of blood given by the intravenous route (Mackay *et al.,* 2002). These 10 animals have been under study for approximately 3 years and have remained healthy with minimal concentrations of viral RNA in plasma and normal CD4 cell counts. The vaccine viral DNA persisted in lymph nodes, and the animals developed CMI responses that were indistinguishable from the effects of Δ*vpu*$SHIV_{PPC}$ described above. In the present study, we examined two new parameters of infection with Δ*vpu*Δ*nef*$SHIV_{PPC}$. First, we report on use of the infectious DNA of the agent as inoculum, and second, we evaluated the effects of intracerebral inoculation of this DNA into animals. This route of inoculation into experimental animals has been deemed essential by the FDA as one of the criteria of safety of any live vaccine.

RESULTS AND DISCUSSION

Two juvenile pigtailed macaques (identification numbers CD3T and CD20) were inoculated intracerebrally with 100 μl of plasmid DNA (1 μg/μl) containing the proviral genome of Δ*vpu*Δ*nef*$SHIV_{PPC}$. One week later, infectious virus was rescued from PBMC, and viral RNA was found in plasma, as indicated by real-time RT-PCR (Fig. 1A). This indicated that systemic infection had been initiated after initial intracerebral infection. Plasma virus peaked at an average of $10^{5.1}$ eq/ml at 3 wk p.i. and declined to $10^{2.9}$ eq/ml by 6 weeks; it remained low but detectable (<1000 eq/ml) through 4.3 months of infection. Viral RNA was also present in all CSF samples (with peak values at 6520 eq/ml in CD3T and 432 in CD20), indicating much lower concentrations than found in the

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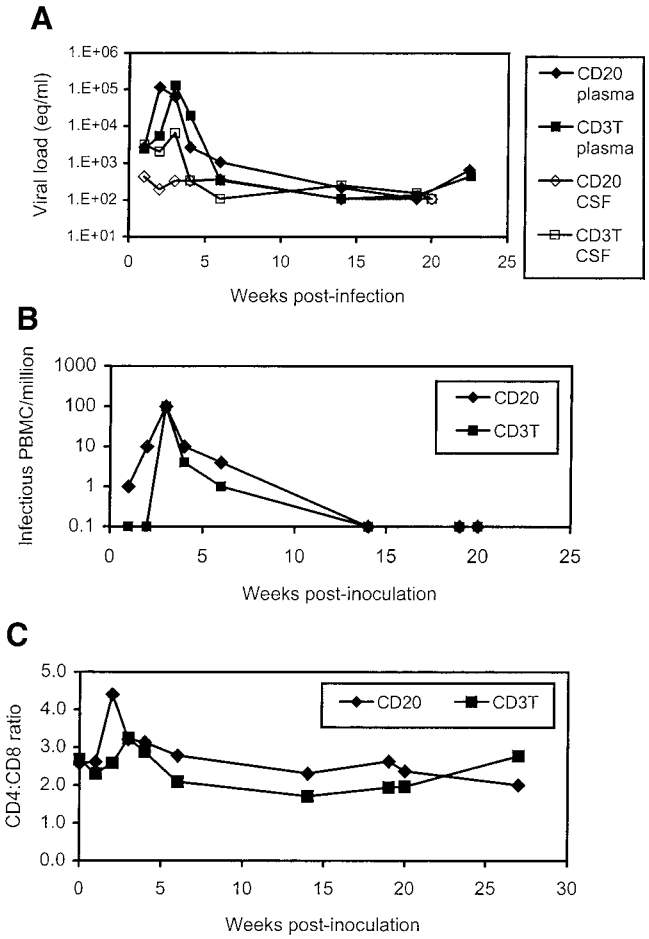

FIG. 1. (A) Cell-free plasma and cerebrospinal fluid viral loads of the two macaques (CD3T and CD20) at multiple time points were determined by real-time RT-PCR, using standards covering six orders of magnitude. The viral loads are shown as viral RNA copies per ml plasma or per ml CSF. (B) Infectious cells in the peripheral blood were monitored over the time course of infection in the macaques with a quantitative culture assay. Dilutions of purified PBMCs were cocultured with C8166 cells and monitored for infection. The results are shown as infectious cells per million PBMCs. (C) Time course of CD4:CD8 ratios in the peripheral blood of the two macaques.

corresponding plasma samples (Fig. 1A). The ratio of plasma viral RNA to CSF viral RNA at 3 weeks was 190 for macaque CD20 and 20 for macaque CD3T. The peak viral loads in these animals were similar to those of six others that had been inoculated orally with live Δ*vpu*Δ*nef*SHIV$_{PPC}$ virus (O. Narayan *et al.,* unpublished observations).

Infectious peripheral blood mononuclear cells (PBMC) were detected during only the first six weeks of infection in both animals and peaked at 100 infectious cells/10^6 PBMCs at 3 weeks p.i. (Fig. 1B). The CD4:CD8 remained above 1.0 (Fig. 1C), in sharp contrast to the results of infection with pathogenic SHIVs that caused nearly total loss of CD4 cells after three weeks (Joag *et al.,* 1997; Liu *et al.,* 1999).

Peripheral blood was drawn, and inguinal peripheral lymph nodes were biopsied at 16 weeks for virus culture and for quantitation of provirus copies and virus RNA expression. PBMC and LN cell suspensions were depleted of CD8 cells and cocultured with C8166 cells that were then observed for virus production. Infectivity was found in LN cells of both animals, but not in the corresponding PBMCs. We also determined the proviral DNA copy number in DNA extracted from flash-frozen portions of the lymph node tissue. Copy numbers were determined as 107 copies/μg for CD20 and 78 copies/μg for CD3T. Total RNA was extracted from other portions of the flash-frozen lymph nodes, and the full-length viral mRNA was detected by real-time RT-PCR using primers and Taqman probe to the *gag* gene. These values were normalized to the *GAPDH* copy number also determined by real-time RT-PCR. The *gag*:*GAPDH* ratios for CD20 and CD3T were 0.17 and 0.37, respectively.

The animals were necropsied at six months postinoculation to determine the distribution and quantity, as well as expression of virus in different organs and tissues. The animals were exsanguinated and perfused with lactated Ringer's solution before any tissue samples were obtained. Samples from 14 regions of the brain and spinal cord were examined. These included frontal, parietal, motor, occipital, and temporal cortices; thalamus; deep white matter; basal ganglia; cerebellum, brainstem, and hippocampus; cervical, thoracic, and lumbar regions of the spinal cord. Portions of each sample were fixed in 4% paraformaldehyde, and paraffin sections were stained with hematoxylin and eosin. Scar tissue along the needle tracks at the site of injection in the brain were detected, and other findings consisted of focal minimal infiltrate in the leptomeninges of CD20, and focal negligible infiltrate in meninges of the thalamus of CD3T. Other lesions in other organ systems are minor.

Real-time PCR was performed on DNA extracted from lymphoid tissues, including peripheral, bronchial, and mesenteric lymph nodes, spleen, thymus, and gut-associated lymphoid tissue from five regions of the GI tract (stomach, jejunum, colon, ileum, duodenum), lung, and from 14 regions of the CNS. The copy numbers obtained were normalized per microgram DNA to give values for equivalent cell numbers. Positive copy numbers of proviral DNA were detected in most of the samples of lymphoid tissues (Fig. 2); four of five GALT samples were positive in CD20, and one of five samples were positive in CD3T. Only 5 of the 14 samples of CNS tissue from CD3T and 3 of the 14 from CD20 were positive for proviral DNA by real-time PCR. The positive CNS samples included the sites of inoculation (right parietal lobes).

To determine whether new rounds of virus replication had occurred in cells in specific locations, we examined tissue DNAs of only those samples with a positive real-time provirus DNA signal for presence of circular proviral DNA using a nested PCR assay for the circle junction. This circular provirus genome is a byproduct of the initial

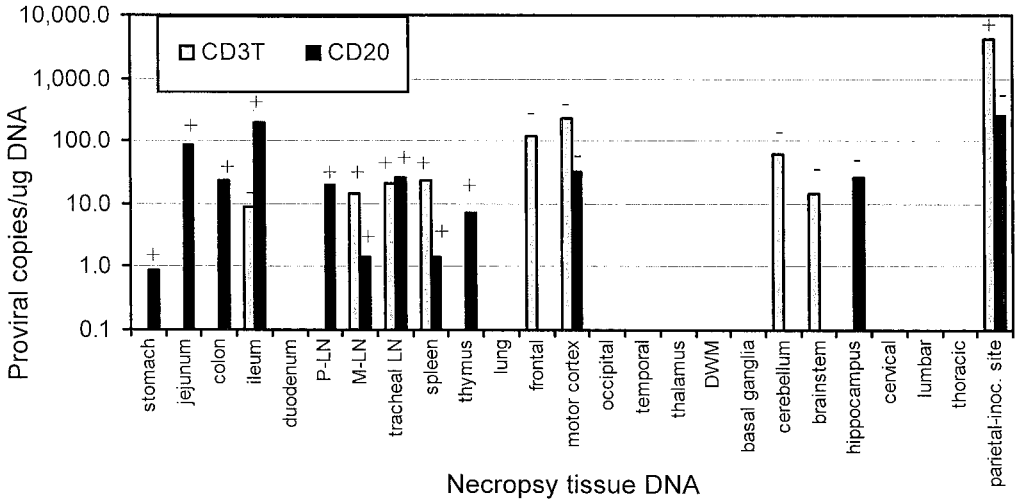

FIG. 2. Proviral DNA copies present in various tissues at necropsy were quantified by real-time PCR. The animals were exsanguinated and perfused prior to obtaining tissue samples. The copy numbers were normalized to 1 μg DNA to indicate the results from equivalent cell numbers. Empy bar spaces represent "not detected." For detection of newly infected cells in the samples, nested PCR for the short-lived circular proviral DNA by-product was also performed on samples testing positive for proviral DNA; the results are shown above the bars (+/−).

step of reverse transcription, and its presence has been interpreted as a sign of newly infected cells (Sharkey *et al.,* 2000). From macaque CD20 all of the lymphoid tissues tested including the GALT were positive for circular DNA; all three of the CNS samples that had viral DNA were negative for circular proviral DNA. From macaque CD3T, in addition to the lymphoid tissues that were nearly all positive for circular DNA, the site of DNA inoculation in the brain was the only CNS region that had circular provirus DNA; the four other brain regions were negative (Fig. 2). Thus, whereas proviral DNA could be detected in some regions of the brain, only lymphoid tissues had signals indicative of ongoing virus replication. That the parietal site of infection (including the needle track) in CD3T was positive, but that of CD20 was not (needle track not found), could potentially be related to macrophages attracted to the site of tissue trauma, which then could be productively infected.

Viral mRNA was detected in the necropsy specimens by real-time RT-PCR for the *gag*-containing full-length transcripts using RNA extracted from lymphoid tissues, selected brain regions, and the site of DNA inoculation. All of the lymphoid tissues tested had a positive signal in the *gag* reactions, and when normalized to the *GAPDH* signal to give a signal per constant cell number, we found that the mesenteric LN and spleen had the highest virus mRNA signals and that the other lymphoid tissues and infection site had close to 10- to 100-fold lower viral mRNA signals (Fig. 3). RNA signal was not detected in the CNS samples except at the injection site of the two

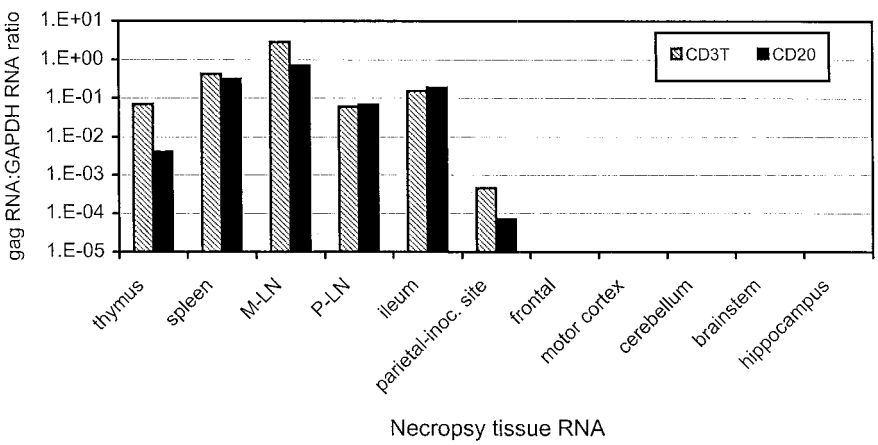

FIG. 3. The presence of viral mRNA in tissues obtained at necropsy was detected by a sensitive real-time RT-PCR assay. The animals were perfused, and tissue samples were flash-frozen in liquid N_2 immediately after removal, as described under Materials and Methods. The indicated lymphoid tissues, the infection site (right parietal lobe), and brain regions with a positive DNA signal (Fig. 2) were chosen for RT-PCR amplification. CD20 frontal, cerebellum, and brainstem, and CD3T hippocampus were not done.

animals. When the *gag*/*GAPDH* RNA signal was normalized to the proviral DNA copies/μg DNA from the same specimens, the ratios were approximately 10^5–10^6 times higher, respectively, in M-LN and spleen than in the infection site of CD3T (ratios: M-LN, 1.97×10^{-1}; spleen, 1.84×10^{-2}; infection site, 1.04×10^{-7}) and CD20 (ratios: M-LN, 4.93×10^{-1}; spleen 2.3×10^{-1}; infection site, 2.72×10^{-7}).

We previously showed that this SHIV deleted in two accessory genes, Δ*vpu*Δ*nef* $SHIV_{PPC}$, functioned well as a vaccine against later challenge with highly pathogenic $SHIV_{KU}$, and that it also was able to reach the brain after oral inoculation of live virus. This study showed that inoculation of infectious DNA of vaccine virus, Δ*vpu*Δ*nef*$SHIV_{PPC}$, intracerebrally into two animals resulted in rapid development of systemic infection in both macaques. The infection was characterized by productive infection in the lymphoid tissues including the thymus, secondary lymphoid tissues in the abdominal cavity, and the GALT, with the most intense replication occurring in the spleen and mesenteric lymph nodes. The CNS also became infected, but only a few regions among the 14 that were sampled had viral DNA. None of the positive CNS samples had circular proviral DNA, with the exception of the injection site (parietal) of one animal in which the needled track was clearly identified. The parietal lobe inoculation site of both animals had detectable viral RNA, whereas none of the other CNS sites with detectable DNA was positive for RNA, suggesting within the limits of sampling errors that only the cells in this region had the ability to support replication of the virus, perhaps due to the stimulation of the monocytes in the area of tissue damage. Thus, among the 28 samples examined from the CNS of the two animals, this was the only site that had viral RNA. Except for focal scarring (with mononuclear cells) of the brain caused by the needle used for inoculation of the DNA, there were very minimal pathological changes in the brains of either of the two animals. The inflammatory cells in the scar at the inoculation site were probably the host cells in which viral RNA localized. Similar to the brain, no pathological changes were observed among the lymphoid tissues, and there was no loss of $CD4^+$ T cells in either of the two animals.

Reconstruction of the events that occurred in the animals, using data from studies on the plasma, the CSF, and the tissues collected at necropsy, suggested that the viral DNA in the inoculum caused infection among cells along the needle path in the brain, as proviral DNA was readily detected in the site of inoculation. The cells taking up the plasmid DNA could have been macrophages lining blood vessel walls or astrocytes. Mononuclear cells from the blood likely responded to the trauma by infiltration of the brain; one scenario of virus dissemination to the systemic lymphoid compartment would be for such monocytes to become infected by contact with resident infected cells and then to transport the virus back out of the CNS and into contact with highly permissive $CD4^+$ T cells.

The results reported here are compatible with known aspects of neuropathogenesis of lentiviral (LV) infections. It is now well established that LV encephalitis in macaques and humans represents end-stage disease and results from explosive virus replication in macrophages in the CNS. Studies on the chronology of neuropathogenic events have shown that the virus invades the CNS early in the systemic infection and viral DNA can readily be found in many regions of the brain and spinal cord. However, examination of brain tissues from animals that were inoculated with pathogenic SHIV and killed sequentially thereafter suggested that the infection was progressive at 3, 10, and 18 days postinoculation, but it eventually became abortive because brains examined at 4 months had only a few regions that had viral DNA. Despite massive virus replication in the lymphoid tissues, with or without severe loss of $CD4^+$ T cells, virus replication in the brain remains at this minimal level for long periods. Increase in replication, accompanied with pathological changes, seems contingent on loss of immunocompetence of the host. It is not known whether the exacerbation of productive replication in the organ at this time results from reactivation of latent virus or from a new invasion from the nonneural sites. In the present study, inoculation of infectious viral DNA directly into the brain did not expedite neuropathogenic effects beyond the type of result that develops following infection initiated by inoculation of the virus at a nonneural site. Since the infection with attenuated virus was not progressive in the lymphoid tissues, an abortive type of infection in the CNS was not unexpected. As previously shown with intracerebral inoculation of pathogenic SIV in rhesus macaques, intracerebral inoculation did not lead to a preferential infection of brain tissue (Boche *et al.,* 1995).

MATERIALS AND METHODS

Construction of Δ*vpu*Δ*nef* SHIV construct and preparation of DNA stock

The construction of $SHIV_{PPC}$ has been described previously (Stephens *et al.,* 1998). Briefly, we used $SHIV_{PPC}$-3, a plasmid derived after macaque passage of SHIV-HXBc2 (Li *et al.,* 1992). A 60-bp deletion was introduced into *vpu,* and a 205-bp deletion of *nef,* which deleted the first 69 amino acids in *nef* (Joag *et al.,* 1998).

Animal care

Animals were individually housed in an Association for Assessment and Accreditation of Laboratory Animal Care international accredited facilities of the University of Kansas Medical Center.

Inoculation of macaques

Two pigtailed macaques, 14–15 months old, were screened serologically for SIV, STLV, and simian retrovirus before inoculation. They were deeply sedated with ketamine, and holes were drilled in the skull over the right hemisphere, approximately 1 cm off midline, in the region of the parietal cortex. The drill bit did not have contact with the dura mater of the meninges. Infectious plasmid preparations of Δ*vpu*Δ*nef* SHIV (100 μl of a 1 μg/μl preparation) were injected into the brain using a 1-in. 27 g needle.

Blood and lymph node samples

Blood samples were collected under ketamine sedation in heparin for infectious virus studies and in EDTA for quantification of viral RNA by RT-PCR. Blood samples were collected weekly for the first month, and periodically until necropsy at 27 weeks p.i. Complete blood counts (CBCs) were performed to monitor leukocyte populations, hematocrit values, and hemoglobin concentrations. Samples were processed as previously described. Briefly, PBMCs were separated from the buffy coats of centrifuged blood and mononuclear cells obtained by centrifugation on gradients of Ficoll–Paque (Pharmacia Biotech, Piscataway, NJ). Cerebrospinal fluid was collected aseptically from the cisterna magna at the time of sedation for blood drawing. Two inguinal lymph nodes were obtained from each animal at 16 weeks p.i. under ketamine anesthesia. One node was used for preparation of cell suspension for biological studies, and the other node was flash-frozen in liquid nitrogen immediately as a source for RNA and DNA.

Cell cultures

The human T cell line C8166 was used as the indicator line to measure virus infectivity. These cells were cultured at a concentration of 10^6/ml in RPMI medium [RPMI 1640 (Life Technologies, Rockville, MD) with 10 mM HEPES buffer (pH 7.3), 50 mg of gentamicin/ml, 50 mM 2-mercaptoethanol, and 2 mM glutamine] with 10% fetal bovine serum (FBS). PBMC and LN cell suspensions were depleted of CD8 T cells as described previously (Joag *et al.,* 1996) and cocultivated with indicator C8166 cells for rescue of infectious virus.

Fluorescence-activated cell sorting analysis

Samples of whole blood were reacted with fluorescent-tagged monoclonal antibodies to $CD3^+$, $CD4^+$, T, and $CD8^+$ (Dako, Carpinteria, CA), as previously described (Wyand *et al.,* 1999). The cells were analyzed on a fluorescence-activated cell counter FACSCalibur System (Becton-Dickinson, San Jose, CA).

Assessment of virus infectivity

Infectivity in PBMC was measured by inoculation of serial 10-fold dilutions of the cell suspensions, starting with 10^6 cells, into 24-well tissue culture plates containing 10^5 indicator C8166 T cells and incubated at 37°C for 7 days. Cells and supernatant fluid in 100 μl from each well were then transferred to another plate, fresh indicator cells added, and the cultures incubated for another 7 days. Cultures were observed for development of syncytial cytopathic effects (CPE). Results were expressed as the number of infectious cells per 10^6 PBMC.

Assessment of viral RNA in plasma

Plasma virus RNA loads were performed on RNA extracted from 500–1000 μl EDTA-anticoagulated plasma. Virus was pelleted from clarified plasma for 1 h at 4°C, at 20,000 *g.* Virus pellets were resuspended in 140 μl PBS, and the RNA was extracted with the QIAgen viral RNA minikit (Qiagen, Valencia, CA). RNA samples were subjected to real-time RT-PCR using *gag* primers and a Taqman probe (5′ fluorescence reporter dye 6-carboxyfluorescein and 3′ quencher dye 6-carboxytetramethylrhodamine) (Hofmann-Lehmann *et al.,* 2000) using an ABI Prism 7700 Sequence Detection System (Foster City, CA). Thermal cycling conditions consisted of 50°C for 2 min for UNG; 60°C for 30 min; 95°C for 10 min; followed by 44 cycles of 95°C for 15 s and 60°C for 30 s. Prime RNAse inhibitor was used in the reactions (7.5 U, Brinkmann-Eppendorf, Westbury, NY), and reaction volumes were 25 μl. Viral RNA equivalents were calculated for 1 ml plasma or CSF. The minimum level of detection for RNA was 18 copies.

Viral messenger RNA quantitation

Inguinal lymph nodes were biopsied at 16 weeks p.i. and flash frozen in liquid nitrogen until processing, as previously described. Necropsy tissue samples were obtained at 27 weeks p.i. Viral *gag* mRNAs were quantified using real-time RT-PCR from total RNA isolated from peripheral lymph nodes or necropsy tissues. *Gag* mRNA was determined using the Taqman probe and primers as described above for the plasma viral RNA determinations during 44 cycles of RT-PCR (ABI). As a measure of cellular mRNA levels, the *GAPDH* mRNA copy numbers in the lymph node RNA samples were also determined by a real-time RT-PCR Taqman assay over 40 cycles (ABI). mRNA numbers based on the use of constant standards were determined in the *gag* mRNA assays, and as the amplification efficiencies of the *gag* and *GAPDH* targets can be considered essentially equal [differences in the slopes (ΔS) of the standard curves was within 0.2], the *gag* mRNA levels were normalized to the cellular *GAPDH* mRNA number.

Proviral DNA content in biopsy and necropsy samples

DNA was extracted from flash-frozen peripheral LN or necropsy samples using Qiagen DNA reagents. DNA copy number was determined by real-time PCR using the same *gag* primers and Taqman probe and the Taqman Universal PCR Mastermix (Applied Biosystems) in duplicate 25-μl reactions in the ABI PRISM 7700 Sequence Detection System. Proviral copy numbers were normalized to the quantity of total tissue DNA used in the reaction. DNA real-time conditions were 50°C for 2 min, 95°C for 10 min, followed by 44 cycles of 95°C for 15 s, 60°C for 30 s. Serial 10-fold dilutions of cloned SHIV *gag* plasmid over six orders of magnitude were used as standards. The minimum detectable level of proviral DNA was 30 copies.

Detection of newly infected cells by circle junction nested PCR

A nested set of primers was designed using the Prime program (Genetics Computer Group, 1999) to span the LTR of the circularized Δ*vpu*Δ*nef* SHIV proviral DNA (the circle junction), with the outer primers positioned upstream and downstream of the SIV LTR sequence. These circular molecules are a dead-end byproduct of the reverse transcription step occurring in newly infected cells and can be used as a marker of newly infected cells (Sharkey *et al.,* 2000). The primers were modified from previous reports to avoid the *nef* deletion region in this virus. The outer primers were cirjntP (5′GGGGTATCAGTGAGGCCAAA 3′) and cirjntQ (5′GCACTAAAGGAGCTAAGACCGAA 3′), resulting in a product of 1347 bp from a 1-LTR circle, and the nested primers included the upstream primer inside the LTR (cirjntC: 5′CGCTCTGTATTCAGTCGCTC3′), and the second downstream from the LTR (cirjntD: 5′AGTTTCTCACGCCCATCTC3′), resulting in a product of 562 bp. The positive control reaction consisted of total DNA extracted from acutely infected CEM cells in culture.

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REFERENCES

Boche, D., Gray, F., Chakrabarti, L., Hurtrel, M., Montagnier, L., and Hurtrel, B. (1995). Low susceptibility of resident microglia to simian immunodeficiency virus replication during the early stages of infection. *Neuropathol. Appl. Neurobiol.* **21,** 535–539.

Genetics Computer Group (GCG). (1999). Madison Wisc. Wisconsin Package Version 10.2.

Hofmann-Lehmann, R., Swenerton, R. K., Liska, V., Leutenegger, C. M., Lutz, H., McClure, H. M., and Ruprecht, R. M. (2000). Sensitive and robust one-tube real-time reverse transcriptase-polymerase chain reaction to quantify SIV RNA load: Comparison of one- versus two-enzyme systems. *AIDS Res. Hum. Retroviruses* **16,** 1247–1257.

Joag, S. V., Adany, I., Li, Z., Foresman, L., Pinson, D. M., Wang, C., Stephens, E. B., Raghavan, R., and Narayan, O. (1997). Animal model of mucosally transmitted human immunodeficiency virus type 1 disease: Intravaginal and oral deposition of simian/human immunodeficiency virus in macaques results in systemic infection, elimination of CD4+ T cells, and AIDS. *J. Virol.* **71,** 4016–4023.

Joag, S. V., Li, Z., Foresman, L., Stephens, E. B., Zhao, L. J., Adany, I., Pinson, D. M., McClure, H. M., and Narayan, O. (1996). Chimeric simian/human immunodeficiency virus that causes progressive loss of CD4+ T cells and AIDS in pig-tailed macaques. *J. Virol.* **70,** 3189–3197.

Joag, S. V., Liu, Z. Q., Stephens, E. B., Smith, M. S., Kumar, A., Li, Z., Wang, C., Sheffer, D., Jia, F., Foresman, L., Adany, I., Lifson, J. D., McClure, H. M., and Narayan, O. (1998). Oral immunization of macaques with attenuated vaccine virus induces protection against vaginally transmitted AIDS. *J. Virol.* **72,** 9069–9078.

Kumar, A., Lifson, J. D., Li, Z., Jia, F., Mukherjee, S., Adany, I., Liu, Z., Piatek, M., Sheffer, D., McClure, H. M., and Narayan, O. (2001). Sequential immunization of macaques with two differentially attenuated vaccines induced long-term virus-specific immune responses and conferred protection against AIDS caused by heterologous simian human immunodeficiency virus (SHIV89.6P). *Virology* **279,** 241–256.

Lamb-Wharton, R., Joag, S. V., Stephens, E. B., and Narayan, O. (1997). Primate models of AIDS vaccine development. *AIDS* **11,** S121–S126.

Li, J., Lord, C. I., Haseltine, W., Letvin, N. L., and Sodroski, J. (1992). Infection of monkeys with a chimeric HIV-1/SIVmac virus that expresses the HIV-1 envelope glycoproteins. *J. Acquir. Immune Defic. Syndr.* **5,** 639–646.

Liu, Z. Q., Muhkerjee, S., Sahni, M., McCormick, D. C., Leung, K., Li, Z., Gattone, V. H., Tian, C., Doms, R. W., Hoffman, T. L., Raghavan, R., Narayan, O., and Stephens, E. B. (1999). Derivation and biological characterization of a molecular clone of SHIV(KU-2) that causes AIDS, neurological disease, and renal disease in rhesus macaques. *Virology* **260,** 295–307.

Mackay, G. A., Niu, Y., Liu, Z. Q., Mukherjee, S., Li, Z., Adany, I., Buch, S., Zhuge, W., McClure, H. M., Narayan, O., and Smith, M. S. (2002). Presence of intact *vpu* and *nef* genes in non-pathogenic SHIV is essential for acquisition of pathogenicity of this virus by serial passage in macaques. *Virology* **295,** 133–146.

Sharkey, M. E., Teo, I., Greenough, T., Sharova, N., Luzuriaga, K., Sullivan, J. L., Bucy, R. P., Kostrikis, L. G., Stevenson, M., Veryard, C., Davaro, R. E., Cheeseman, S. H., Daly, J. S., Bova, C., Ellison, R. T. I., Mady, B., Lai, K. K., Moyle, G., Nelson, M., Gazzard, B., and Shaunak, S. (2000). Persistence of episomal HIV-1 infection intermediates in patients on highly active anti-retroviral therapy. *Nat. Med.* **6,** 76–81.

Silverstein, P., Mackay, G. A., Mukherjee, S., Li, Z., Piatak, M., Jr., Lifson, J. D., Narayan, O., and Kumar, A. (2000). Pathogenic simian/human immunodeficiency virus $SHIV_{KU}$ inoculated into immunized macaques caused infection, but virus burdens progressively declined with time. *J. Virol.* **74,** 10489–10497.

Stephens, E. B., Mukherjee, S., Liu, Z. Q., Sheffer, D., Lamb, W. R., Leung, K., Zhuge, W., Joag, S. V., Li, Z., Foresman, L., Adany, I., and Narayan, O. (1998). Simian-human immunodeficiency virus (SHIV) containing the nef/long terminal repeat region of the highly virulent SIVsmmPBj14 causes PBj-like activation of cultured resting peripheral blood mononuclear cells, but the chimera showed no increase in virulence. *J. Virol.* **72,** 5207–5214.

Wyand, M. S., Manson, K., Montefiori, D. C., Lifson, J. D., Johnson, R. P., and Desrosiers, R. C. (1999). Protection by live, attenuated simian immunodeficiency virus against heterologous challenge. *J. Virol.* **73,** 8356–8363.