Generation of the Pathogenic R5-Tropic Simian/Human Immunodeficiency Virus SHIV_{AD8} by Serial Passaging in Rhesus Macaques^{†‡}

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A new pathogenic R5-tropic simian/human immunodeficiency virus (SHIV) was generated following serial passaging in rhesus macaques. All 13 animals inoculated with SHIV_{AD8} passaged lineages experienced marked depletions of CD4⁺ T cells. Ten of these infected monkeys became normal progressors (NPs) and had gradual losses of both memory and naïve CD4⁺ T lymphocytes, generated antiviral CD4⁺ and CD8⁺ T cell responses, and sustained chronic immune activation while maintaining variable levels of plasma viremia (10² to 10⁵ RNA copies/ml for up to 3 years postinfection [p.i.]). To date, five NPs developed AIDS associated with opportunistic infections caused by *Pneumocystis carinii*, *Mycobacterium avium*, and *Campylobacter coli* that required euthanasia between weeks 100 and 199 p.i. Three other NPs have experienced marked depletions of circulating CD4⁺ T lymphocytes (92 to 154 cells/ μ l) following 1 to 2 years of infection. When tested for coreceptor usage, the viruses isolated from four NPs at the time of their euthanasia remained R5 tropic. Three of the 13 SHIV_{AD8}-inoculated macaques experienced a rapid-progressor syndrome characterized by sustained plasma viremia of >1 $\times$ 10⁷ RNA copies/ml and rapid irreversible loss of memory CD4⁺ T cells that required euthanasia between weeks 19 and 23 postinfection. The sustained viremia, associated depletion of CD4⁺ T lymphocytes, and induction of AIDS make the SHIV_{AD8} lineage of viruses a potentially valuable reagent for vaccine studies.

Simian immunodeficiency virus (SIV)/macaque models of AIDS have been extensively used as surrogates for human immunodeficiency virus type 1 (HIV-1) in studies of virus-induced immunopathogenesis and vaccine development. As is observed for the HIVs recovered from a majority of individuals during the asymptomatic phase of their infections, pathogenic SIVs utilize the CCR5 coreceptor to enter their CD4⁺ T lymphocyte targets *in vivo* (36). This leads to the elimination of memory CD4⁺ T cells circulating in the blood and residing at effector sites (gastrointestinal [GI] tract, mucosal surfaces, and lung), particularly during acute HIV and SIV infections (5, 29, 32, 49). In contrast to naturally occurring SIVs and HIVs, SIV/HIV chimeric viruses (simian/human immunodeficiency viruses [SHIVs]) were constructed in the laboratory by inserting a large segment of the HIV genome, including the *env* gene, into the genetic backbone of the molecularly cloned SIV_{mac239} (44). SHIVs were developed because they expressed the HIV envelope glycoprotein and could be used in vaccine

experiments to evaluate neutralizing antibodies (NAbs) elicited by HIV-1 gp120 immunogens. The commonly used pathogenic SHIVs generated high levels (10⁷ to 10⁸ RNA copies/ml) of plasma viremia and induced an extremely rapid, systemic, and nearly complete depletion of the entire CD4⁺ T cell population, resulting in death from immunodeficiency beginning at 3 months postinoculation (23, 26, 41). Unlike SIVs, however, these pathogenic SHIVs exclusively targeted CXCR4-expressing CD4⁺ T cells during infections of rhesus monkeys (36). Despite their extraordinary virulence, most vaccine regimens (naked DNA, peptides, proteins, inactivated virions, recombinant modified vaccinia virus Ankara (MVA), and DNA prime/recombinant viral-vector boosting) were effective in controlling intravenous (i.v.) and mucosal X4-tropic SHIV challenges (1, 3, 33, 42, 46). When it became apparent that the same vaccination strategies that were effective in suppressing pathogenic SHIVs failed to control SIV infections, concerns were raised about whether X4 SHIVs were appropriate surrogates for HIV in vaccine experiments (13).

The unusual biological properties of the X4 SHIVs plus the discrepant outcomes of SIV and X4 SHIV vaccine experiments have become a driving force for developing CCR5-utilizing (R5) SHIVs. Although several clade B and clade C R5-tropic SHIVs have been constructed (7, 15, 21, 30, 38), the SHIV_{SF162} lineage viruses are the best-characterized and most widely used R5 SHIVs (20). They have been employed in microbicide (10), neutralizing monoclonal antibody (MAb) passive-transfer (16, 17), and vaccination (2) studies.

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In the aftermath of the failed STEP HIV vaccine trial, there was general consensus that additional SIVs and SHIVs should be developed, particularly for use as heterologous challenge viruses in vaccine studies (12). With this goal in mind, we report the generation of a new pathogenic R5-tropic SHIV bearing the *env* gene from the HIV-1_{Ada} isolate (14). HIV-1_{Ada} was selected because it is a prototypical macrophage-tropic strain (8), uses CCR5 for cell entry (53), and has the potential for eliciting NAbS against HIV-1 gp120, and we had previously constructed a full-length infectious molecular clone (pHIV-1_{AD8}) (48). Based on previous experience in obtaining pathogenic X4-tropic SHIVs, serial passaging in macaques, treated with an anti-CD8 MAb at the time of virus inoculation, was used to expedite the adaptation of R5-SHIV sequences in a nonhuman primate host. Of the 13 animals inoculated with *in vivo*-passaged SHIV_{AD8#2} (see below) and its immediate derivatives, 10 exhibited a normal-progressor (NP) phenotype, sustaining gradual depletions of both memory and naïve CD4⁺ T cells from the circulation and memory CD4⁺ T cells at an effector site (lung) while maintaining variable viral-RNA loads (10² to 10⁵ RNA copies/ml) for up to 3 years postinfection (p.i.). Five of these monkeys developed immunodeficiency with associated opportunistic infections requiring euthanasia. Three other NPs currently have total CD4⁺ T cell counts of 92 to 154 cells/μl plasma after 1 to 2 years of infection. The remaining 3 of the 13 SHIV_{AD8}-inoculated macaques experienced a rapid-progressor (RP) clinical course and were euthanized between weeks 19 and 23 p.i. because of intractable diarrhea and marked weight loss. The sustained viremia, associated depletion of CD4⁺ T lymphocytes, and induction of AIDS make the SHIV_{AD8} lineage of viruses a potentially valuable reagent for vaccine studies.

MATERIALS AND METHODS

Construction of SHIV_{AD8}. SHIV_{AD8} contains the *env* gene from the R5-tropic HIV-1_{Ada} (14)-derived molecular clone pHIV_{AD8} (48). A 3.04-kb segment from pHIV_{AD8}, including a portion of the *vpr* gene and the entire *tat*, *rev*, *vpu*, and *env* genes, was PCR amplified using the forward primer TGAACTTATGGGGA TACTTGGGC, which begins at nucleotide 141 of the AD8 *vpr* gene, allowing the incorporation of a unique EcoRI site, located 21 nucleotides downstream from the primer, into the PCR product. The reverse PCR primer (TCCACCCATAA GCTTATAGCAAAGTCCTTCCAAGCCC) generated a HindIII site adjacent to and encompassing the last 2 nucleotides of the *env* reading frame, as well as a substitution of a Thr for a Leu 3 codons upstream from the *env* termination codon. PCRs were performed using 10 pmol each of the forward and reverse primers, Platinum PCR SuperMix High Fidelity (Invitrogen), and 1 μl of pHIV_{AD8} in a final volume of 50 μl. The reaction mixtures were heated to 94°C for 2 min, followed by 30 cycles of 94°C for 20 s, 59°C for 30 s, and 70°C for 3 min and a 7-min extension at 70°C. The PCR product was gel extracted using a Qiaquick gel extraction kit (Qiagen) and digested with EcoRI and HindIII, and the resulting 3.04-kb restriction fragment was cloned directly into the previously described and similarly digested pSHIV_{DH12} (45) to generate pSHIV_{AD8}. DNA sequencing of the entire 3.04-kb insert in pSHIV_{AD8} was conducted to verify that no spurious changes had been introduced during the PCR amplification and cloning.

Preparation of SHIV_{AD8} virus stocks. HeLa cells were transfected with 25 μg of pSHIV_{AD8}, and the virus present in the supernatant at 48 h was pelleted in an ultracentrifuge and resuspended in RPMI 1640 medium as previously described (52). Stocks of the cloned SHIV_{AD8} were prepared by infecting PM1 cells (31) or concanavalin A (ConA)-activated rhesus monkey peripheral blood mononuclear cells (PBMC) with the HeLa-derived SHIV_{AD8}, as previously described (23, 24), and pooling the supernatant media at the times of peak reverse transcriptase (RT) production from both infections.

SHIV_{AD8} stock 2 (SHIV_{AD8#2}) was prepared from PBMC and bone marrow (BM), spleen, and lymph node (LN) samples collected from macaque CK1G on

TABLE 1. Infection of rhesus macaques with SHIV_{AD8#2} and immediate derivatives

Animal	Inoculum
CJ8B.....	SHIV _{AD8#2}
CK15.....	Blood transfusion from CJ8B (wk 60)
CJ58.....	Blood transfusion from CJ8B (wk 60)
CE8J.....	Lymph node virus ^a (SHIV _{AD8#2LN} , 3.2 × 10 ⁵ TCID ₅₀) from CJ8B (wk 59)
CJ35.....	Lymph node virus (SHIV _{AD8#2LN} , 3.2 × 10 ⁵ TCID ₅₀) from CJ8B (wk 59)
CJ3V.....	PBMC virus ^b (SHIV _{AD8#2PBMC} , 5.9 × 10 ⁴ TCID ₅₀) from CK15 + CJ58 (wk 4)
CK5G.....	PBMC virus (SHIV _{AD8#2PBMC} , 5.9 × 10 ⁴ TCID ₅₀) from CK15 + CJ58 (wk 4)
DB99.....	Blood transfusion from CJ8B (wk 117) + CK15 (wk 57) + CJ58 (wk 57)
DA1Z.....	Blood transfusion from CJ8B (wk 117) + CK15 (wk 57) + CJ58 (wk 57)
A4E008.....	Blood transfusion from DA1Z (wk 1) + DB99 (wk 1)
DA4W.....	Blood transfusion from DA1Z (wk 1) + DB99 (wk 1)
CL5A.....	SHIV _{AD8#2} passaged <i>in vitro</i> for 30 days (SHIV _{AD8#2.d30} , 4.3 × 10 ⁵ TCID ₅₀)
CL98.....	SHIV _{AD8#2} passaged <i>in vitro</i> for 30 days (SHIV _{AD8#2.d30} , 4.3 × 10 ⁵ TCID ₅₀)

^a Lymph node virus: SHIV_{AD8} derivative prepared from the supernatant medium collected from cocultures of lymph node suspensions plus PBMC, recovered from animal CJ8B at week 59 p.i., and PBMC from uninfected rhesus monkeys.

^b PBMC virus: SHIV_{AD8} derivative prepared from the supernatant medium collected from cocultures of PBMC, recovered from the indicated infected animals at week 4 p.i., and PBMC from uninfected rhesus monkeys.

day 6 p.i. Cell suspensions from axillary, inguinal, iliac, and mesenteric LNs, PBMC, and BM were cocultivated with PBMC from uninfected animals; the culture supernatants were monitored daily for RT activity, pooled, and designated SHIV_{AD8#2}. The infectious titer of SHIV_{AD8#2} was 1.5 × 10³ tissue culture infective doses (TCID₅₀)/ml, as determined in rhesus macaque PBMC.

SHIV_{AD8} lymph node virus (SHIV_{AD8LN}) was prepared from supernatant medium collected from cocultures of lymph node suspensions plus PBMC recovered from animal CJ8B at week 59 p.i. (Table 1) and PBMC from uninfected rhesus monkeys. The infectious titer of SHIV_{AD8LN} was 6.4 × 10³ TCID₅₀/ml, as determined in rhesus macaque PBMC.

SHIV_{AD8} PBMC virus (SHIV_{AD8PBMC}) was prepared from supernatant medium collected from cocultures of PBMC recovered and pooled from animals CK15 and CJ58 at week 4 p.i. (Table 1) and PBMC from uninfected rhesus monkeys. The infectious titer of SHIV_{AD8PBMC} was 1.1 × 10⁴ TCID₅₀/ml, as determined in rhesus macaque PBMC.

SHIV_{AD8#2.d30} was prepared by infecting ConA-stimulated pig-tailed macaque (PT) PBMC with SHIV_{AD8#2}. Fresh ConA-stimulated PT PBMC were added to the infected cultures on days 10 and 20, and the supernatant medium collected on day 30, designated SHIV_{AD8#2.d30}, had an infectious titer of 8.5 × 10⁴ TCID₅₀/ml, as determined in rhesus macaque PBMC.

Virus replication assay in rhesus monkey PBMC. The preparation and infection of rhesus monkey PBMC have been described previously (25). Briefly, PBMC stimulated with concanavalin A and cultured in the presence of recombinant human interleukin-2 (IL-2) were spinoculated (1,200 × g for 1 h) (37) with virus normalized for RT activity. Virus replication was assessed by RT assay of the culture supernatant as described above.

Animal experiments. Rhesus macaques (*Macaca mulatta*) were maintained in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals (9) and were housed in a biosafety level 2 facility; biosafety level 3 practices were followed. Phlebotomies, i.v. virus inoculations, euthanasia, and tissue sample collections were performed as previously described (11). Bronchoalveolar lavage (BAL) fluid lymphocytes were prepared from uninfected and infected animals using a pediatric bronchoscope (Olympus BF3C40; Olympus America, Inc., Melville, NY), as previously described (22).

Serial *in vivo* passaging of SHIV_{AD8} was initiated by transferring whole blood (10 ml) and BM (2 ml) to a recipient animal previously treated with the anti-CD8⁺ T cell-depleting MAb cM-T807 (10 mg/kg of body weight) on days -1 and +3 p.i. In subsequent passages, spleen, LN (axillary, inguinal, iliac, and mesen-

teric), PBMC, and BM cell suspensions were prepared from infected donors at the time of necropsy and transferred (1×10^8 to 3×10^8 mononuclear cells and 1×10^8 to 10×10^8 BM cells) to a new recipient by the i.v., intraperitoneal (i.p.), and BM routes.

Quantitation of proviral-DNA and plasma viral-RNA levels. The number of viral-DNA copies in PBMC was measured by quantitative DNA PCR (45). Viral-RNA levels in plasma were determined by real-time reverse transcription-PCR (ABI Prism 7700 sequence detection system; Applied Biosystems, Foster City, CA) as previously reported, using reverse-transcribed viral RNA in plasma samples from SIV_{mac239}-inoculated rhesus macaques (11).

Lymphocyte immunophenotyping and data analysis. EDTA-treated blood samples and BAL fluid lymphocytes were stained for flow cytometric analysis as described previously (34, 36), using combinations of the following fluorochrome-conjugated MAb: CD3 (fluorescein isothiocyanate [FITC] or phycoerythrin [PE]), CD4 (PE, peridinin chlorophyll protein-Cy5.5 [PerCP-Cy5.5], or allophycocyanin [APC]), CD8 (PerCP or APC), CD28 (FITC or PE), CD95 (APC), and Ki-67 (FITC or PE). All antibodies were obtained from BD Biosciences (San Diego, CA), and samples were analyzed by four-color flow cytometry (FACS-Calibur; BD Biosciences Immunocytometry Systems). Data analysis was performed using CellQuest Pro (BD Biosciences) and FlowJo (TreeStar, Inc., San Carlos, CA). For Ki-67 staining, cells were fixed with fluorescence-activated cell sorter (FACS) lysing solution (Becton Dickinson), treated with FACS permeabilization buffer 2 (Becton Dickinson), and stained with Ki-67 MAb or a control isotype IgG1. In this study, naïve CD4⁺ T cells were identified by their CD95^{low} CD28^{high} phenotype, whereas memory CD4⁺ T cells were CD95^{high} CD28^{high} or CD95^{high} CD28^{low} in the CD4⁺ small lymphocyte gate (36, 39).

Intracellular-cytokine assays. Stimulation was performed on frozen lymphocytes as described previously (40). Freshly thawed lymphocytes were resuspended (10^6 /ml) in RPMI medium supplemented with antibiotics and glutamine. Anti-CD28 conjugated to Alexa 594-PE was used for costimulation. Staphylococcus enterotoxin B (1 µg/ml; Sigma-Aldrich, St. Louis, MO) was used to stimulate T cells mitogenically through the T cell receptor as a positive control. A negative control (cells treated only with costimulatory anti-CD28) was included in every experiment. Peptides used to stimulate SIV-specific T cells were 15 amino acids (aa) in length, overlapping by 11 amino acids, and encompassed SIV_{mac239} Gag (New England Peptide, Gardner, MA). The concentration of each peptide was 2 µg/ml for stimulations, which were performed in the presence of brefeldin A (BFA) (1 µg/ml; Sigma-Aldrich, St. Louis, MO) for 16 h at 37°C. All cells were surface stained with the dead-cell exclusion dye Aqua Blue (Invitrogen Corp., Carlsbad CA), followed by staining with anti-CD3 Alexa 700 (BD Biosciences), anti-CD4 Cy5.5-PE (eBioscience Inc., San Diego, CA), anti-CD8 Pacific Blue (BD Biosciences), and anti-CD95 Cy5-PE (BD Biosciences). The cells were then fixed, permeabilized, and stained with anti-gamma interferon (IFN-γ) Cy7-PE (BD Biosciences), anti-IL-2 APC (BD Biosciences), tumor necrosis factor (TNF) FITC (BD Biosciences), and Mip1-β PE (BD Biosciences). SIV-specific CD8 T cell responses are reported as the frequency of memory CD8 T cells, gated by characteristic light scatter properties; then as Aqua Blue⁻, CD3⁺, CD8⁺, CD4⁻, or CD95⁺; and by production of either TNF or Mip1-β. All data are reported after background subtraction.

Virus neutralization assays. Autologous plasma samples (1:20 dilution) from SHIV_{AD8}-infected macaques were incubated with (i) the same uncloned SHIV_{AD8} derivative used for inoculation or (ii) the SHIV_{AD8} isolated from PBMC at week 4 p.i. (for monkeys CJ58 and CK15) in quadruplicate in 96-well flat-bottom culture plates in a total volume of 50 µl for 1 h at 37°C. Prechallenge plasma samples from each animal served as controls. Freshly trypsinized TZM-bl cells (50×10^4 (1.5×10^4 in 150 µl Dulbecco's modified Eagle's medium [DMEM] containing 20 µg/ml DEAE dextran) were added to each well, and the cultures were maintained in a 37°C incubator for 28 h. The amount of virus-induced luciferase activity, measured as relative light units (RLU), present in cell lysates was determined as previously described (51), and the average neutralization activity for each plasma sample was determined. The average number of RLU for the prechallenge plasma controls ranged from 1×10^5 to 2×10^5 . Any sample resulting in a 50% reduction of luciferase activity compared to that obtained with the uninfected control sample was considered positive for NAb. To determine neutralizing-antibody titers, 40 µl of diluted virus, sufficient to generate the desired numbers of RLU, was mixed with 10 µl of appropriately diluted plasma samples in a 96-well plate and incubated for 1 h at 37°C. TZM-bl cells were added, cultures were maintained for an additional 28 h, and intracellular luciferase activity was measured as described above.

Coreceptor utilization assays. Freshly trypsinized TZM-bl cells (1×10^4 per well) in 135 µl DMEM containing 10% fetal calf serum (FCS) and DEAE dextran (15 µg/ml) were seeded in flat-bottom 96-well plates. Twenty-five microliters of coreceptor antagonists (AD101 against CCR5, AMD3100 against

CXCR4, or both, at final concentrations ranging from 0.1 nM to 1,000 nM) was added to each well. Following incubation for 1 h at 37°C, 10 TCID₅₀ of replication-competent virus, determined in TZM-bl cells as previously described, in 40 µl was added to each well. After 24 h of incubation at 37°C, luciferase activity was determined. The percent infectivity reported was derived from the mean of quadruplicate assays.

To generate 293T cell-derived SHIV_{AD8} pseudotyped viruses, two separate plasmids were constructed. The first (pNLenv1) contained a frameshifted mutation in the leader peptide region of gp120 (43). Plasmids expressing the SHIV_{AD8}(RIG⁺) and SHIV_{AD8}(RIG⁻) *env* genes [pCMV-AD8(RIG⁺) and AD8(RIG⁻)] were generated by reverse transcription-PCR of plasma viral RNA, collected from macaque DB99 at the time of euthanasia, and subcloning into NotI and (newly created) XbaI sites of the pCMVbeta expression plasmid (Clontech, Palo Alto, CA). Both plasmids [pNLenv1 and pCMV-AD8(RIG) in a 5:1 ratio] were cotransfected into 293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The titers of pseudotyped-virus preparations were determined, and they were assayed for coreceptor usage 48 h following infection of TZM-bl cells, as described for replication-competent virus.

RESULTS

Construction of a CCR5-tropic SHIV. We previously reported the construction of a full-length infectious HIV-1 molecular clone (pHIV-1_{AD8}) derived from the prototypical macrophage-tropic CCR5-utilizing HIV-1_{Ada} isolate (14, 48). A SHIV expressing the *env* gene from pHIV_{AD8} was obtained by inserting the 3.04-kb EcoRI-to-HindIII DNA fragment (including a portion of *vpr* and the entire *tat*, *rev*, *vpu*, and *env* genes) into the genetic background of pSHIV_{DH12} (45), as described in Materials and Methods. The resulting molecular clone, pSHIV_{AD8}, directed the production of progeny virions following the transfection of HeLa cells. Virus stocks were prepared by infecting PM1 cells or ConA-stimulated rhesus PBMC with virions pelleted from HeLa cell transfection culture supernatants.

It is not generally appreciated how daunting it is to generate an R5-tropic SHIV able to maintain detectable levels of set-point viremia, exclusively target memory CD4⁺ T cells, and induce immunodeficiency in inoculated rhesus monkeys. Simply replacing orthologous SIV sequences with a DNA segment including a CCR5-utilizing HIV-1 *env* gene does not usually result in a SHIV exhibiting robust replication kinetics *in vivo* and a disease-inducing phenotype. This was, in fact, the case for SHIV_{AD8}: levels of plasma viremia following virus inoculation (1 ml of undiluted virus by the i.v., i.p., and BM routes) were promptly and durably suppressed, and the numbers of memory CD4⁺ T lymphocytes did not change appreciably, as shown for a representative infected animal (CJ7H) in Fig. 1a. To be certain that we were on the right track with respect to the targeting and elimination of memory, not naïve, CD4⁺ T cells *in vivo*, a second macaque (CJ9F) was treated with the CD8⁺ T lymphocyte-depleting MAb cM-T807 24 h prior to SHIV_{AD8} inoculation, as well as on days 3 and 6 post-virus infection, to promote a vigorous *in vivo* infection. Unlike untreated macaque CJ7H, the levels of plasma viral RNA in monkey CJ9F rapidly rose to 3.8×10^7 copies/ml by day 10 p.i. and were associated with a rapid and irreversible decline of circulating memory CD4⁺ T cells (Fig. 1b). In contrast, the numbers of naïve CD4⁺ T lymphocytes in animal CJ9F were maintained in the 600- to 800-cell/µl range during this period. This result, therefore, confirmed that SHIV_{AD8} could sustain high virus loads and preferentially target the memory CD4⁺ T

FIG. 1. Infectivity of the original SHIV_{AD8} in rhesus monkeys. Macaques CJ7H (a) and CJ9F (b) were inoculated with 1 ml of undiluted SHIV_{AD8} by the i.v., i.p., and BM routes. Macaque CJ9F was treated with the depleting anti-CD8 MAb cM-T807 as indicated.

cell subset *in vivo*, but only in an animal with a compromised immune system.

In vivo passaging of SHIV_{AD8}. The prompt control of plasma viremia and the nonpathogenic phenotype of SHIV_{AD8} observed in untreated macaques were reminiscent of the infectivity patterns observed with first-generation X4-tropic SHIVs (28, 44). We therefore initiated serial animal-to-animal passaging of SHIV_{AD8} with macaque CJ9F as the “founder” infected monkey (Fig. 2a). This approach had previously been used to generate X4 SHIVs exhibiting more robust replicative and pathogenic properties (26, 41). Unfortunately, *in vivo* serial passaging of virus to optimize infectivity is an empirical and stochastic process. One never knows when or if an R5 SHIV has acquired an augmented replicative phenotype. The ultimate proof that such a change has occurred requires the inoculation of additional animals and waiting several months to assess the resultant viral replication kinetics and CD4⁺ T cell dynamics.

The strategy employed was to maximize the emergence of disease-inducing SHIV variants, putatively present in an in-

creasingly genetically diverse virus population, by serially transferring large numbers of infected cells by i.v., i.p., and BM routes into recipient animals previously treated with an anti-CD8 depleting MAb. As indicated in Fig. 2a, whole blood and bone marrow cells were transferred from macaque CJ9F to macaque H681 by these three routes. In subsequent passages, cell suspensions were prepared from spleen, LN (axillary, inguinal, iliac, and mesenteric), PBMC, and BM cells collected at the time of necropsy, as described in Materials and Methods. With one exception (macaque CJ7F), the depleting anti-CD8 MAb was administered to a recipient animal on days –1 and +3 p.i. to facilitate unrestricted replication *in vivo*. Animal CJ7F did not receive anti-CD8 MAb at the time of virus transfer to investigate the possibility that SHIV_{AD8} had acquired improved replication properties *in vivo* following the initial two *in vivo* passages. Because this was not the case (its plasma viral-RNA loads had declined to 360 RNA copies/ml at week 10 p.i.), macaque CJ7F was treated with anti-CD8 MAb at week 13 p.i. and sustained an immediate burst of virus production that reached 1.4×10^6 RNA copies/ml of plasma at week

FIG. 2. Serial animal-to-animal passage of SHIV_{AD8}. (a) Passage history of SHIV_{AD8} and origin of SHIV_{AD8}#2. (b) Rhesus monkey PBMC were infected with SHIV_{AD8} or the passaged SHIV_{AD8}#2 virus stock, normalized for RT activity. CPM, counts per minute.

FIG. 3. SHIV_{AD8#2} induces sustained plasma viremia and loss of CD4 T cells in an inoculated rhesus macaque. Plasma viremia and the percentage of BAL fluid CD4⁺ T cells (a) or the absolute numbers of circulating CD4⁺ T cells (b) in rhesus macaque CJ8B inoculated intravenously with SHIV_{AD8#2} are shown. RT-PCR, reverse transcription-PCR.

14 p.i. CJ7F was euthanized at week 15 p.i., and cell suspensions were prepared as described above and transferred by the i.v., i.p., and BM routes into macaque CK1A, previously treated with anti-CD8 MAb (Fig. 2a). Following the fifth *in vivo* passage, macaque CK1G was euthanized on day 6 p.i., and cell suspensions, prepared at the time of necropsy, were cocultivated with ConA-stimulated PBMC from uninfected rhesus monkeys as described in Materials and Methods; the culture supernatants were monitored for the presence of reverse transcriptase activity, pooled, and designated SHIV_{AD8#2}.

Inoculation of rhesus macaques with SHIV_{AD8#2} and its immediate derivatives resulted in sustained plasma viremia and loss of CD4⁺ T lymphocytes. To ascertain whether serial passaging of SHIV_{AD8} *in vivo* had resulted in the acquisition of improved replicative properties, ConA-stimulated rhesus monkey PBMC were infected with SHIV_{AD8#2} or the starting SHIV_{AD8} virus preparation, both normalized for RT activity. As shown in Fig. 2b, SHIV_{AD8#2} replicated to much higher levels in cultured macaque PBMC than the original SHIV_{AD8}. To determine whether this improved infectivity of SHIV_{AD8#2} for rhesus PBMC was correlated with augmented replication in an animal not treated with the depleting anti-CD8 MAb, macaque CJ8B was inoculated i.v. with 1.5×10^4 TCID₅₀ of SHIV_{AD8#2}. As shown in Fig. 3a, this monkey experienced a marked but transient depletion of memory CD4⁺ T cells in BAL specimens during the acute infection and maintained detectable levels of plasma viremia. Because animal CJ8B subsequently experienced a decline in the total circulating CD4⁺ T lymphocyte population from 565 to 175 cells/μl at week 56 p.i. (Fig. 3b), whole blood or virus propagated *ex vivo* from CJ8B lymph node suspensions (lymph node virus [SHIV_{AD8LN}]) was inoculated into four additional macaques (CK15, CJ58, CE8J, and CJ35) (Fig. 4). Four other animals (DB99, DA1Z, A4E008, and DA4W) received blood transfusions, and two (CJ3V and CK5G) were inoculated with PBMC coculture virus (SHIV_{AD8PBM}) derived from monkeys CK15 and CJ58 (Fig. 4). In addition, because it was unknown at the time of its preparation whether SHIV_{AD8#2} had acquired augmented *in vivo* infectivity properties, SHIV_{AD8#2} was propagated for an additional 30 days *ex vivo* in macaque PBMC as described in Materials and Methods. Because the resulting derivative, designated SHIV_{AD8#2.d30}, exhibited robust infectivity in both pigtailed and rhesus macaque PBMC (data not

shown), it was inoculated intravenously into two rhesus monkeys (CL5A and CL98) (Fig. 4). The inocula used to infect rhesus monkeys with SHIV_{AD8#2} and its immediate derivatives are listed in Table 1. None of these monkeys received the depleting anti-CD8 MAb.

Ten of the 13 animals infected with SHIV_{AD8#2} or its immediate derivatives experienced an NP clinical course characterized by set-point virus loads that varied widely (from less than 10^3 to more than 10^5 RNA copies/ml) and a gradual depletion of circulating CD4⁺ T lymphocytes (Fig. 5a and b). Transient, and in some cases quite significant, losses of memory CD4⁺ T cells in BAL samples was a common finding during the acute infection (Fig. 5c). The loss of circulating CD4⁺ T lymphocytes in the 10 SHIV_{AD8#2}-infected NPs affected both memory and naïve subsets (Fig. 6). With one exception (monkey CJ35), these animals sustained depletions of circulating memory CD4⁺ T cells to the 200-cell/μl level by week 100. NPs also experienced increased memory CD4⁺ T lymphocyte turnover, as monitored by Ki-67 expression, particularly during the first 10 weeks and the final stages of the infection (see Fig. S1 in the supplemental material). The loss of naïve CD4⁺ T lymphocytes in NP monkeys was even more profound. By week 80 p.i., this subset had declined to below 100 cells/μl in all of the animals (Fig. 6b). At the time of their euthanasia, five NPs (CJ8B, CE8J, CJ3V, CK15, and CL98) had only 1, 3, 6, 12, and 68 circulating naïve CD4⁺ T cells/μl, respectively. We previously reported that SIVsmE543-infected

FIG. 4. SHIV_{AD8#2} and its immediate derivatives cause immunodeficiency in rhesus macaques. The dashed arrows indicate virus transfer by blood transfusion. The thick arrows indicate LN or PBMC specimens used to generate virus stocks by coculturing with PBMC from uninfected donors. †, euthanized animals.

FIG. 5. Total CD4⁺ T lymphocytes are gradually lost in normal progressors following infection with SHIV_{AD8#2} and its immediate derivatives. The levels of plasma viremia (a), absolute numbers of peripheral CD4⁺ T cells (b), and percentages of BAL fluid CD4⁺ T cells (c) are shown. The five normal progressors that developed AIDS and were euthanized are indicated (†).

NPs had also experienced a marked loss of naïve CD4⁺ T cells as early as 20 weeks p.i. (35). It was therefore not unexpected that NP SHIV_{AD8}-infected monkeys might also sustain a depletion of their naïve CD4⁺ T cell subset.

Three of the 13 macaques inoculated with SHIV_{AD8#2} and its immediate derivatives became RPs, requiring euthanasia between weeks 19 and 23 p.i. because of anorexia, intractable diarrhea, and marked weight loss (Fig. 7). Virus set points in the RPs exceeded 10^7 RNA copies/ml, memory CD4⁺ T cells in BAL specimens rapidly and irreversibly declined, and at the time of death, all of the animals had sustained marked losses of circulating CD4⁺ T cells.

Immune responses to SHIV_{AD8}. In the context of its use as a challenge virus in vaccine experiments, it was important to show that SHIV_{AD8} elicited both cellular and humoral immune responses during infections of rhesus monkeys. Therefore, anti-SHIV_{AD8} Gag-specific CD8⁺ T lymphocyte re-

sponses were measured by flow cytometry for 6 of the 10 NPs by intracellular staining of cells expressing TNF- α and/or IFN- γ following stimulation with a 15-mer peptide pool spanning SIV_{mac239} Gag. The levels of virus-specific CD8⁺ T cells in this group of rhesus monkeys ranged from 0.33 to 1.68% during the second year of their infection (see the table in the supplemental material). A similar analysis of Gag-specific responses in memory CD4⁺ T cells at these times in the same animals indicated that 0.90 to 2.90% expressed TNF- α and/or IFN- γ (see the table in the supplemental material).

NABs were detected in several of the NPs during the course of their infections (Fig. 8). The seven macaques evaluated had been inoculated with SHIV_{AD8#2} or two immediate derivatives (SHIV_{AD8#2LN} and SHIV_{AD8#2PBM}). Plasma neutralizing activity directed against the same virus used for animal challenge was evaluated in monkeys CJ8B, CE8J, CJ35, CJ3V, and

FIG. 6. Marked depletion of naive and memory CD4⁺ T lymphocytes characterizes long-term SHIV_{AD8} infection in NP rhesus monkeys. Absolute numbers of memory CD4⁺ T cells (a) and naive CD4⁺ T cells (b) in 10 normal-progressor macaques during 200 weeks of SHIV_{AD8} infection are shown. †, euthanized animals.

CK5G. The neutralization sensitivity of autologous virus (SHIV_{AD8#2PBM}) was monitored using plasma collected from PBMC of macaques CK15 and CJ58 (Fig. 4). The time of appearance of neutralization activity varied widely (week 20 to week 78 p.i.) and was generally correlated with levels of set-point viremia. In the three macaques producing the highest levels of anti-SHIV_{AD8} NAb, the actual 50% inhibitory concentration (IC₅₀) neutralization titers determined by limiting plasma dilution were 1:159 (CJ8B at week 89), 1:102 (CJ58 at week 30), and 1:143 (CE8J at week 52).

Coreceptor usage by SHIV_{AD8} lineage viruses. The *env* gene of SHIV_{AD8} was derived from the prototypical macrophage-tropic HIV-1_{AdA}, previously shown to use CCR5 for cell entry (53). When tested in a TZM-bl entry assay with inhibitors that specifically target CXCR4 or CCR5, the original SHIV_{AD8}, SHIV_{AD8#2} (data not shown), and SHIV_{AD8#2LN} exclusively utilized CCR5 (see Fig. S2 in the supplemental material). The marked depletion of circulating naive CD4⁺ T cells in all SHIV_{AD8} NPs (Fig. 6b) raised the possibility that a coreceptor switch had occurred, enabling these viruses to enter and eliminate naive CD4⁺ T cells, which express high levels of surface CXCR4, but not CCR5. Accordingly, virus was recovered from three NPs (CK15, CE8J, and CL98) immediately prior to euthanasia. When tested for coreceptor usage, the viruses isolated from all three NPs remained R5 tropic (see Fig. S2 in the supplemental material), indicating that the loss of naive CD4⁺ T cells was not due to direct virus-induced cell killing.

As noted earlier, three monkeys infected with SHIV_{AD8#2} derivatives exhibited an RP phenotype. By week 10 p.i., these macaques (DB99, A4E008, and CL5A) had experienced massive loss of memory CD4⁺ T cells in samples collected by BAL (Fig. 7c) but had little change in the number of circulating naive CD4⁺ T lymphocytes (data not shown). However, by week 19 p.i., the levels of total CD4⁺ T cells in the blood had declined significantly in all three RPs (Fig. 7b), raising again the possibility that coreceptor usage might have changed. To assess a possible coreceptor switch, virus was collected from RP monkeys DB99 and A4E008 at the time of euthanasia and evaluated in the TZM-bl assay with specific CXCR4 and CCR5 inhibitors. As shown in Fig. 9a, blocking the entry of SHIV_{AD8-DB99} required both inhibitors, whereas SHIV_{AD8-A4E008} was inhibited only by the

CCR5 inhibitor. This result indicates that SHIV_{AD8-DB99} had acquired the capacity to use CXCR4 during its infection of macaque DB99 and that SHIV_{AD8-A4E008} had remained R5 tropic.

Reverse transcription-PCR cloning and sequencing of *env* genes amplified from the plasma of macaque DB99 at the time of its euthanasia revealed that 28 of 29 recovered clones contained a 3-aa insertion (RIG) located 2 residues upstream of the GPGR sequence in the crown of the gp120 V3 region (Fig. 9b). A similar analysis of the *env* gene from virus circulating in monkey A4E008 revealed a different 3-aa insertion (HIG) at the same location in its V3 loop. The V3 loop sequences amplified from the plasma of both animals at week 2 p.i. did not contain any insertion. The gp120 region amplified from the third RP (macaque CL5A) at the time of euthanasia contained no insertion (Fig. 9b).

One of the 28 viral-DNA clones amplified from macaque DB99 plasma at the time of euthanasia containing the RIG insertion in V3 and the single clone simultaneously obtained from this animal lacking the V3 insertion were used to prepare pseudotyped virus for testing in the entry assay, as described in Materials and Methods. As shown in Fig. 9c, the V3 RIG insertion conferred usage of both CCR5 and CXCR4 coreceptors on SHIV_{psAD8(RIG+)} compared to the exclusive utilization of CCR5 by SHIV_{psAD8(RIG-)}, which lacks the gp120 V3 insertion.

SHIV_{AD8}-infected macaques developed immunodeficiency. The clinical statuses and disease outcomes of all 13 animals inoculated with SHIV_{AD8#2} and its immediate derivatives during a 2- to 3-year observation period are presented in Table 2. As noted above, 10 of these 13 macaques were NPs and experienced gradual and irreversible depletions of both memory and naive CD4⁺ T lymphocyte subsets (Fig. 6). Five of these animals were euthanized with symptoms of AIDS, and 3 additional NPs currently have CD4⁺ T cell counts ranging from 92 to 154 cells/μl plasma (Table 2). Histopathological studies performed on specimens collected at the time of necropsy revealed the presence of *Pneumocystis carinii*, *Mycobacterium avium*, and *Campylobacter coli* infections in individual macaques (see Fig. S3 in the supplemental material). In addition, 3 of the 13 R5-SHIV-infected monkeys experienced an RP syndrome characterized by sustained plasma viremia of

FIG. 7. Patterns of virus replication and CD4⁺T cell dynamics in SHIV_{AD8} rapid progressors. The levels of plasma viremia (a), absolute numbers of peripheral CD4⁺ T cells (b), and percentages of BAL fluid CD4⁺ T cells (c) are shown. †, euthanized animals.

$>1 \times 10^7$ RNA copies/ml; rapid and irreversible loss of memory CD4⁺ T cells in the blood and at an effector site (BAL); and intractable diarrhea, anorexia, and weight loss requiring euthanasia between weeks 19 and 23 p.i.

DISCUSSION

The results presented clearly show that the generation of a pathogenic R5-SHIV was not a trivial undertaking. Animal-to-animal passaging eventually gave rise to SHIV_{AD8#2}, possessing greatly augmented infectivity for rhesus PBMC compared to the starting SHIV_{AD8} construct. Although it was not appreciated at the time, SHIV_{AD8#2} had also acquired improved *in*

vivo properties, as evidenced by its and its immediate derivatives' capacity to cause fatal immunodeficiency in 8 of 13 inoculated rhesus monkeys (Fig. 4 and Table 2). The most consistent and distinguishing property of the passaged SHIV_{AD8} family of viruses during infections of rhesus macaques was the slow and unremitting loss of both memory and naïve CD4⁺ T cells (Fig. 6), a pattern of depletion observed in all 10 NPs. Surprisingly, and in contrast to both SIVmac and SIVsmE lineages, the pace of CD4⁺ T lymphocyte decline was not correlated with plasma virus loads. Although the geometric mean plasma viral-RNA level at week 50 in the SHIV_{AD8}-infected monkey cohort was 1.7×10^3 RNA copies/ml, the set-point virus loads varied widely in the 10 infected animals

FIG. 8. Neutralizing-antibody activities detected in normal-progressor macaques following infection with SHIV_{AD8#2} or its immediate derivatives. Plasma samples (1:20 dilution) from the indicated SHIV_{AD8}-infected macaques were incubated in quadruplicate for 1 h at 37°C with the virus isolates shown in parentheses and then used as an inoculum to infect TZM-bl cells. The luciferase activity present in cell lysates at 28 h p.i. was measured, and the average percent neutralization activity in plasma at each time point was determined. Prechallenge plasma samples served as negative controls and baselines for zero neutralizing-antibody activity.

FIG. 9. Coreceptor utilization of SHIV_{AD8} derivatives isolated from rapid progressors. (a) TZM-bl cells were infected in quadruplicate with viruses (SHIV_{AD8-DB99} and SHIV_{AD8-A4E008}) recovered from rapid progressors DB99 and A4E008, respectively, in the presence of the indicated amounts of the small-molecule coreceptor inhibitors AD101 (CCR5), AMD 3100 (CXCR4), or both. SIV_{mac239} and SHIV_{DH12RCL-7} were also analyzed as representative R5-tropic and dual-tropic viruses, respectively. The luciferase activities present in cell lysates 24 h p.i. were measured, and percent infectivities were determined in the absence or presence of coreceptor inhibitors. (b) gp120 sequences from the N-terminal V3 regions of SHIV_{AD8} variants, recovered from three RP animals, were aligned with the starting SHIV_{AD8} V3 loop. The V3 regions of the R5-tropic SHIV_{SF162P3} and its SHIV_{SF162-BR24N} derivative, which also emerged in an RP, are included in the alignment. (c) Coreceptor utilization of virus, pseudotyped with Envs present in RP DB99 at the time of necropsy, containing or lacking the 3-aa RIG V3 loop insertion.

TABLE 2. Clinical and pathological findings in rhesus monkeys infected with SHIV_{AD8#2} and its immediate derivatives

Animal	Clinical data/pathological findings
CJ8B	Euthanized (wk 199); uncontrolled diarrhea; wt loss
CK15	Euthanized (wk 112); <i>P. carinii</i> pneumonia
CJ58	Total CD4 ⁺ T cells, 154/mm ³ (wk 111)
CE8J	Euthanized (wk 117); uncontrolled diarrhea, <i>C. coli</i> enteritis
CJ35	Total CD4 ⁺ T cells, 270/mm ³ (wk 129)
CJ3V	Euthanized (wk 135); uncontrolled diarrhea; typhlocolitis
CK5G	Total CD4 ⁺ T cells: 101/mm ³ (wk 101)
DB99	Euthanized (wk 23); rapid progressor
DA1Z	Total CD4 ⁺ T cells, 545/mm ³ (wk 65)
A4E008	Euthanized (wk 20); rapid progressor
DA4W	Total CD4 ⁺ T cells, 92/mm ³ (wk 64)
CL5A	Euthanized (wk 19); rapid progressor
CL98	Euthanized (wk 100); disseminated <i>M. avium</i>

(1.6×10^2 to 1.5×10^5 RNA copies/ml). This variability was also observed in pairs of animals inoculated with identical SHIV_{AD8#2} derivatives (*viz.* CK15 and CJ58, and DB99 and DA1Z). An extreme example of the nonlinkage between viral-RNA levels and CD4⁺ T cell loss with SHIV_{AD8} occurred with animal CK5G, which had 43 and 42 circulating naïve and memory CD4⁺ T cells/ μ l, respectively, at week 86 p.i. and a plasma viral load of only 5.4×10^2 RNA copies/ml. During the chronic phase of SHIV_{AD8} infections, the loss of naïve CD4⁺ T cells was more rapid and more marked than the depletion of the memory subset, as was previously observed in SIVsmE543-infected animals (35) (Fig. 6). By week 80, for example, NPs had sustained an 87 to 93% loss of naïve CD4⁺ T cells from their preinoculation levels, whereas the depletion of memory cells was significant, but not as pronounced. The dissociation of plasma virus loads and CD4⁺ T cell loss is reminiscent of the previously reported infection of pig-tailed macaques with SIVl'hoest and SIVsun (4). In that study, 8 of 12 infected animals developed immunodeficiency over a 5-year period while maintaining set-point viremia between 10^2 and 10^3 RNA copies/ml.

We do not presently understand why naïve CD4⁺ T lymphocytes are lost in SHIV_{AD8} NPs. Based on coreceptor expression, this T cell subset expresses CXCR4, not CCR5, on its surface and should therefore be refractory to infection by R5-tropic SHIVs and virus-induced cell killing. An assessment of the coreceptor utilization status of late-stage viruses recovered from SHIV_{AD8} NPs, in fact, revealed that a coreceptor switch had not occurred in these animals (see Fig. S2 in the supplemental material). Although a dissociation between viral-RNA levels and memory/naïve CD4⁺ T cell loss was observed, the NPs did experience increased memory CD4⁺ T lymphocyte turnover (see Fig. S1 in the supplemental material), even in animals with very low plasma virus loads. Activation-induced proliferation and killing of memory CD4⁺ T cells during the lengthy chronic SHIV_{AD8} infection might therefore be responsible for driving the differentiation of naïve CD4⁺ lymphocytes into memory cells and impose an unsustainable drain on this CD4⁺ T cell subset. It is also possible that SHIV_{AD8} infection of rhesus macaques negatively affects naïve CD4⁺ T lymphocyte homeostasis in the thymus, thereby impeding the differ-

entiation or emigration of this T cell subset. It has also recently been reported that the loss of naïve CD4⁺ T cells during SIVsmE543 infections was associated with the presence of autoreactive antibodies to CD4⁺ T lymphocytes, platelets, double-stranded DNA, and phospholipid (27). Increased numbers of circulating IgG-coated CD4⁺ T cells were observed in that study, and the levels of autoreactive antibodies were correlated with the extent of naïve CD4⁺ T cell depletion.

Approximately 20% of rhesus monkeys infected with SIVmac/SIVsm lineage viruses become RPs, experiencing persistently high virus set points, rapid and complete losses of memory CD4⁺ T cells, undetectable or transient antiviral antibody responses, and early onset (3 to 6 months p.i.) of symptomatic disease (6). Despite losing virtually all of their memory CD4⁺ T lymphocytes, SIV RPs, at the time of death, usually maintain preinoculation levels of naïve CD4⁺ T cells (35). This was not the case for SHIV_{AD8} RPs. Although all three experienced early and massive depletions of memory CD4⁺ T cells, two of the infected macaques had lost virtually all of their naïve CD4⁺ T cells at the time of euthanasia. In one of these animals (DB99), the virus recovered at the time of euthanasia, as well as a virus pseudotyped with an Env possessing the RIG insertion in the V3 loop, had acquired the capacity to infect cells expressing CXCR4 (Fig. 9a and c). Interestingly, coreceptor switching has been previously reported to occur during RP infections of macaques inoculated with a different R5-tropic SHIV, SHIV_{SF162P3} (18, 19, 47). In one of the SHIV_{SF162P3} coreceptor-switching events, the insertion of two positively charged amino acids (HR) immediately upstream of the V3 loop GPGR crown (Fig. 9b) was shown to confer X4 tropism (18). In the case of SHIV_{AD8-DB99}, a 3-aa (RIG) insertion, also located in the N-terminal V3 stem and which increased the net charge of the V3 loop from +3 to +5, was responsible for the acquisition of CXCR4 usage. The insertion of HIG at the same location of the SHIV_{AD8-A4E008} V3 region did not affect the net charge and did not confer tropism for CXCR4-expressing cells.

Independent and unrecognized cross-species transmissions and spread of SIVsm at different U.S. primate facilities during the 1970s contributed to the emergence of SIVmac and SIVsmE660 lineages with distinctive replicative and pathogenic phenotypes. The serial passaging of SHIV_{AD8} in rhesus monkeys described here also resulted in an AIDS-inducing primate lentivirus with its own characteristic properties. First, in contrast to commonly used pathogenic SIVs, SHIV_{AD8#2} and its immediate derivatives generated sustained but, as previously noted, highly variable set-point virus loads in NPs. Similarly variable viral loads were also observed in eight rhesus monkeys inoculated with four independent SHIV_{AD8} stocks prepared from macaques CK15, CE8J, CL98, and CJ58 at the time of their euthanasia (data not shown). Profound depletions of both memory and naïve CD4⁺ T cells, which accompany relatively low virus set points (geometric mean level, 1.7×10^3 RNA copies/ml) in NPs, is a second property that distinguishes the R5-tropic SHIV_{AD8} from pathogenic SIVs. Finally, unlike SIVs, SHIV_{AD8} RPs experience an initial loss of memory CD4⁺ T lymphocytes and a later rapid deletion of naïve CD4⁺ T cells prior to death, which in one animal occurred following a CCR5-to-CXCR4 coreceptor switch. Based on the results shown in Fig. 4 and Table 2, we plan to use and distribute

SHIV_{AD8#2LN}, SHIV_{AD8#2PBM}, or the SHIVs recovered from NPs at the time of euthanasia (SHIV_{AD8-CL98}, SHIV_{AD8-CK15}, or SHIV_{AD8-CE8J}) as challenge viruses in vaccine experiments. Animals inoculated with cell-free preparations of the last group of viruses have experienced variable but sustained plasma viremia associated with a gradual but significant CD4⁺ T cell loss during 30 weeks of infection. Some of these macaques have developed a rapid-progressor clinical course.

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