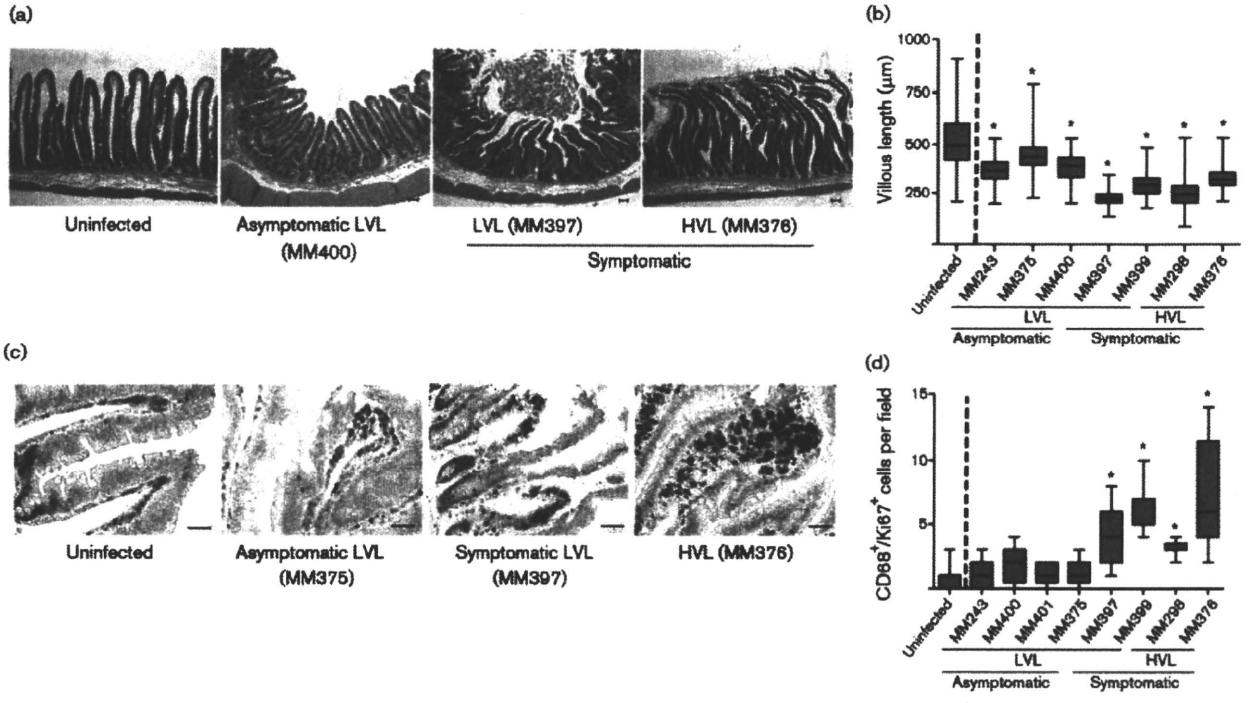

Fig. 3. Villous length in jejunum and counts of activated macrophages in the small intestine at the time of euthanasia in SHIV-KS661-infected rhesus macaques. (a) H&E-stained sections of jejunum of representative uninfected, Asym LVL, Sym LVL and HVL macaques. Bars, 200 μm. (b) Comparison of villous length in uninfected and infected macaques. The lengths of at least 100 villi were measured in each macaque. Statistical analysis was performed using Student's *t*-test for the data from four uninfected and each infected macaque (*, $P<0.0001$). Data for MM299, MM338, MM339 and MM401 were not available. (c) Ki67 and CD68 staining in the small intestine of representative uninfected, Asym LVL, Sym LVL and HVL macaques. Brown staining indicates $Ki67^+$ cells and blue staining indicates $CD68^+$ cells. Bar, 50 μm. (d) Comparison of $CD68^+$ $Ki67^+$ cell counts in uninfected and infected macaques. The numbers of $CD68^+$ $Ki67^+$ cells were enumerated in at least ten fields of the tissues at a magnification of 200×. Statistical analysis was performed using Student's *t*-test for the data from seven uninfected and each infected macaque (*, $P<0.0001$). Data for MM299, MM338 and MM339 were not available.

an LVL. The LVL macaques had much stronger antibody responses than the HVL macaques (Table 1). SHIV-89.6P is easily controlled by the antibody response (Montefiori *et al.*, 1998). SHIV-KS661, which shares its genetic origin with SHIV-89.6P, might be strongly affected by the antibody response. Virus replication during the primary phase clearly occurred later in the intrarectally inoculated macaques than in the intravenously inoculated macaques. Therefore, this delay might contribute to the continuous and strong antibody response in the intrarectally inoculated macaques, consequently resulting in a low viral load in most of the intrarectally inoculated macaques.

The purpose of this study was to elucidate why LVL macaques experience diarrhoea and wasting. A comparison of circulating $CD4^+$ T-cell counts (Fig. 2a) and relative levels of naïve T-cells (Fig. 2b) in LVL macaques did not reveal a substantial difference between Sym LVL (which showed diarrhoea and wasting) and Asym LVL (which were healthy) macaques. The villous length in the intestine also did not affect the level of malignancy of the disease condition, as all infected monkeys showed significant villous atrophy, suggesting a high sensitivity to infection itself. However, Sym LVL and HVL macaques exhibited two findings that Asym LVL macaques did not: (i) $CD4^+$ cell reduction in intestinal and lymphoid tissues (Fig. 2c, d), a hallmark of AIDS; and (ii) abnormal innate immune activation, which was reflected by an increased number of activated macrophages within the intestines (Fig. 3c, d). Ki67 serves as a proliferation marker and proliferation of macrophages may seem unlikely. However, there are some reports about local macrophage proliferation in inflammation sites, indicating the infiltration of activated macrophages associated with tissue damage (Isbel *et al.*, 2001; Norton, 1999). These observations indicated the existence of immunopathological disorders in the intestines not only in HVL macaques but also in Sym LVL macaques.

Many studies have shown positive correlations between the development of AIDS and some characteristic features in

the intestinal tracts of HIV-1-infected humans and pathogenic SIV- or SHIV-infected monkeys: continuous $CD4^+$ T-cell depletion (Brenchley *et al.*, 2004; Ling *et al.*, 2007), abnormal and chronic immune activation (Brenchley *et al.*, 2006; Hazenberg *et al.*, 2003) and enteropathy (Kotler, 2005). Immune activation (as shown by an increased number of intestinal activated macrophages) and intestinal $CD4^+$ cell depletion in Sym LVL macaques strongly suggest the presence of an AIDS-like disease in this subset of animals. Hence, these results suggest that an AIDS-like intestinal disease can occur in LVL macaques despite their low viral load, as well as in HVL macaques.

Some HIV-1-infected patients experience poor recovery of circulating $CD4^+$ T cells, even when their plasma HIV-1 RNA load is suppressed by HAART (Kaufmann *et al.*, 2003; Marchetti *et al.*, 2006; Piketty *et al.*, 1998). These individuals are called immunological non-responders (Marchetti *et al.*, 2006), and have been found to have increased plasma lipopolysaccharide levels, suggesting that bacteria had been translocated from the intestines into the circulation with concomitant activation of T-cell compartments (Marchetti *et al.*, 2006, 2008). Furthermore, some patients who maintain an undetectable or nearly undetectable plasma viral RNA load in the absence of HAART also develop AIDS disease progression (Madec *et al.*, 2005) and have abnormal immune activation and increased plasma lipopolysaccharide levels (Hunt *et al.*, 2008). These observations may indicate that disease progression in a subset of HIV-1-infected individuals is independent of viraemia. Accordingly, the disease progression under conditions of low viral load that we observed in SHIV-KS661-infected macaques can also occur in HIV-1-infected individuals.

Consistent with the fact that intestinal $CD4^+$ cell depletion triggers mucosal immune dysfunction, a notable difference observed between Sym LVL and Asym LVL macaques was the low $CD4^+$ cell frequency in the intestines of the Sym LVL macaques. We propose that the intestinal $CD4^+$ cells in Sym LVL macaques were not able to recover after intestinal $CD4^+$ cell reduction during the early phases of infection. We reported previously that SHIV-KS661 infection of rhesus macaques caused early intestinal $CD4^+$ T-cell depletion (Fukazawa *et al.*, 2008; Miyake *et al.*, 2006). Although we did not examine the macaques during the early phases of infection, the intestinal $CD4^+$ T cells of both Sym LVL and Asym LVL macaques should have been depleted at this time, as even moderately pathogenic SHIV can cause intestinal $CD4^+$ cell reduction during the early phase of infection (Fukazawa *et al.*, 2008). Therefore, the near-normal frequency of intestinal $CD4^+$ cells in Asym LVL macaques would be the result of $CD4^+$ cell recovery after intestinal $CD4^+$ cell reduction during the early phase of infection. In contrast, intestinal $CD4^+$ cells in Sym LVL macaques may be unable to recover, even though virus replication has been controlled. Similarly, intestinal $CD4^+$ cell recovery was found to be important for halting disease progression in SIVmac239-infected rhesus macaques (Ling *et al.*, 2007). Accordingly, one of the important determinants for disease progression in SHIV-KS661-infected macaques may be $CD4^+$ cell recovery in the intestines.

We further hypothesize that this inappropriately low level of $CD4^+$ cells within the intestines of the SHIV-KS661-infected animals (and phenotypically similar humans) is permissive to the excessive activation of resident tissue macrophages. One implication of these studies is that regulatory T-cell subsets of $CD4^+$ cells may be especially vulnerable to this depletion, thus allowing this macrophage activation in view of the well-known role of regulatory T cells in inhibiting innate immune responses (Maloy *et al.*, 2003). This hypothesis will be important to assess in future studies to understand the pathophysiology in the intestines during the chronic phase of HIV-1 infection.

Taken together, the present results suggest that $CD4^+$ cell reduction and enteropathy can occur in SHIV-KS661-infected rhesus macaques even when the viral load is low. The ability or inability to restore intestinal $CD4^+$ cells may be a key factor determining disease progression, irrespective of virus replication levels in the chronic phase of SHIV-KS661 infection. The reason that the recovery of intestinal $CD4^+$ cells is impeded is unknown, although we can speculate on some possibilities such as the co-existence of other infectious microbial agents or impaired T-cell reconstitution caused by damage during thymopoiesis at an early phase of SHIV infection (Motohara *et al.*, 2006). We demonstrated comparable proviral DNA loads in the examined tissues between Sym and Asym LVL macaques, although the $CD4^+$ cell frequencies in the tissues were clearly reduced in Sym LVL macaques. Therefore, the quantity of provirus per CD4 cell in the tissues of Sym LVL macaques is considered to be relatively higher than that of Asym LVL macaques, and low-level replication that may be undetectable in the plasma viral load might be maintained in Sym LVL but not in Asym LVL macaques. Identifying the mechanisms of poor recovery of intestinal $CD4^+$ cells is needed to understand AIDS pathogenesis, because, as stated above, some HIV-1-infected patients have low $CD4^+$ T-cell counts even when viraemia is controlled. One useful approach is comparative and periodical analysis, including cellular immunology data, of the intestinal tract of the same animals from the early to the chronic phases using Sym LVL and Asym LVL macaques in this SHIV infection macaque model.

METHODS

Virus, animals and sample collection. Highly pathogenic SHIV-KS661 is a molecular clone of SHIV-C2/1 (GenBank accession no. AF217181), which was derived through *in vivo* passages of SHIV-89.6 (Shinohara *et al.*, 1999). The virus stock was prepared from the supernatant of virus-infected CEMx174 and M8166 human lymphoid cell lines.

All rhesus macaques used in this study were treated in accordance with the institutional regulations approved by the Committee for

Experimental Use of Non-human Primates in the Institute for Virus Research, Kyoto University, Japan. All macaques were inoculated with 2×10^3 50 % tissue culture infectious dose of SHIV-KS661 measured with CEMx174. The animal ID numbers, infection route and when they were euthanized are provided in Fig. 1(a).

Blood was collected periodically using sodium citrate as an anti-coagulant and examined by flow cytometry and for quantification of plasma viral RNA load. Tissue samples were obtained at the time of euthanasia and were used for quantification of proviral DNA and histopathology.

Determination of plasma viral RNA and proviral DNA loads. The viral loads in plasma and proviral DNA loads in lymphoid and intestinal tissues were determined by quantitative RT-PCR and quantitative PCR, respectively, as described previously (Motohara *et al.*, 2006). DNA samples were extracted directly from frozen tissue sections of each monkey using a DNeasy Tissue kit (Qiagen) according to the manufacturer's protocol.

Determination of antibody titres. Anti-HIV antibody titres were determined using a commercial particle agglutination kit (Serodia-HIV1/2; Fujirebio). Isolated plasma samples were serially diluted and assayed. The end point of the highest dilution giving a positive result was determined as the titre.

Flow cytometry. Flow cytometry was performed as described previously (Motohara *et al.*, 2006). Briefly, $CD4^+$ T cells were analysed by a combination of fluorescein isothiocyanate (FITC)-conjugated anti-monkey CD3 (clone FN-18; BioSource) and phycoerythrin-conjugated anti-human CD4 (clone NU-TH/I; Nichirei), and subsets of naïve and memory $CD4^+$ cells were analysed by a combination of FITC-conjugated anti-human CD95 (clone DX2; BD Pharmingen) and allophycocyanin-conjugated anti-human CD4 (clone L200; BD Pharmingen). $CD95^-$ $CD4^+$ cells were defined as naïve $CD4^+$ T cells and $CD95^+$ $CD4^+$ cells were defined as memory $CD4^+$ T cells. Labelled lymphocytes were examined on a FACSCalibur analyser using CellQuest software (BD Biosciences).

Histology and immunohistochemistry. Tissue samples were fixed in 4 % paraformaldehyde in PBS at 4 °C overnight and embedded in paraffin wax. Sections (4 μm) were dewaxed using xylene, rehydrated through an alcohol gradient, and stained with H&E. The villous length of the jejunum was measured with a micrometer. At least 40 villi from each section were measured.

For immunohistochemistry, sections were rehydrated and processed for 10 min in an autoclave in 10 mM citrate buffer (pH 6.0) to unmask the antigens, sequentially treated with TBS/Tween 20 (TBST) and aqueous hydrogen peroxide, left at 4 °C overnight or at room temperature for 30 min or 1 h for primary antibody reactions, washed with TBST, incubated at room temperature for 1 h with an Envision+ kit (a horseradish peroxidase-labelled anti-mouse immunoglobulin polymer; Dako), visualized using diaminobenzidine (DAB) substrate (Dako) as a chromogen, rinsed in distilled water, counterstained with haematoxylin and analysed by light microscopy (Biozero BZ-8000: Keyence).

For double staining (CD68 and Ki67) of sections, appropriately processed sections were incubated at room temperature for 1 h with unlabelled anti-Ki67 antibody at a dilution of 1 : 2000, the highly sensitive tyramide amplification step (CSAII; Dako) was performed, the slides were reacted with DAB to visualize the results and incubated with unlabelled anti-CD68 antibody at 4 °C overnight followed by incubation at room temperature for 1 h with Histofine Simple Stain AP (an alkaline phosphatase-labelled anti-mouse immunoglobulin polymer (Nichirei), and the results were visualized with a Blue Alkaline Phosphatase Substrate kit III (Vector Laboratories). Measurements of $CD68^+$ $Ki67^+$ cell counts were performed in ten fields at a magnification of 200 × by light microscopy.

Primary antibodies used in immunohistochemistry were anti-human CD4 (diluted 1 : 30; clone NCL-CD4; Novacastra Laboratories), anti-SIV Nef (diluted 1 : 500; FIT Biotech), anti-human CD68 (diluted 1 : 50; clone KP-1; Dako) and anti-human Ki67 (Ki-S5; Dako).

Statistical analysis. The significance of $CD4^+$ or $CD68^+$ $Ki67^+$ cell frequency measurements and villous length in the jejunum of infected monkeys compared with uninfected monkeys was analysed using an unpaired Student's *t*-test (two-tailed) using GraphPad Prism 4.0E software (Varsity Wave).

ACKNOWLEDGEMENTS

We are grateful to Dr James Raymond for editing the English of this manuscript; to Takahito Kazama, Reii Horiuchi, Noriko Nakajima and Tetsutaro Sata for technical support; to Dr Michael A. Eckhaus for histopathological interpretation; and to Takeshi Kobayashi for critical reading. This work was supported, in part, by Research on HIV/AIDS in Health and Labour Sciences Research Grants from the Ministry of Health, Labour and Welfare, Japan; a Grant-in-Aid for Scientific Research from the Ministry of Education and Science, Japan; a Research Grant for AIDS on Health Sciences focusing on Drug Innovation from the Japan Health Sciences Foundation; and a Program for the Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO) of Japan.

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JOURNAL OF VIROLOGY, May 2010, p. 4769–4781 Vol. 84, No. 9
0022-538X/10/$12.00 doi:10.1128/JVI.02279-09

Generation of the Pathogenic R5-Tropic Simian/Human Immunodeficiency Virus $SHIV_{AD8}$ by Serial Passaging in Rhesus Macaques[▽]†

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Received 28 October 2009/Accepted 31 January 2010

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 (10^2 to 10^5 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/μl) 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 $>1 \times 10^7$ 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 (10^7 to 10^8 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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† Supplemental material for this article may be found at http://jvi.asm.org/.

▽ Published ahead of print on 10 February 2010.

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 (10^2 to 10^5 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 TGAAACTTATGGGGA 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 GCTTATAGCAAAGTCCTTTCCAAGCCC) 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 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 1.5×10^3 tissue culture infective doses ($TCID_{50}$)/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 6.4×10^3 $TCID_{50}$/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 1.1×10^4 $TCID_{50}$/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 8.5×10^4 $TCID_{50}$/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-

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 *in vitro* for 30 days ($SHIV_{AD8\#2.d30}$, 4.3×10^5 $TCID_{50}$)
CL98	SHIVAD8#2 passaged *in vitro* 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.

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 MAbs: 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 (FACSCalibur; 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 Mip-1β. 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) (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 NAbs. 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 $TCID_{50}$ 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 (Clonetech, 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 ($pHIV\text{-}1_{AD8}$) derived from the prototypical macrophage-tropic CCR5-utilizing $HIV\text{-}1_{Ada}$ 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 durable 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

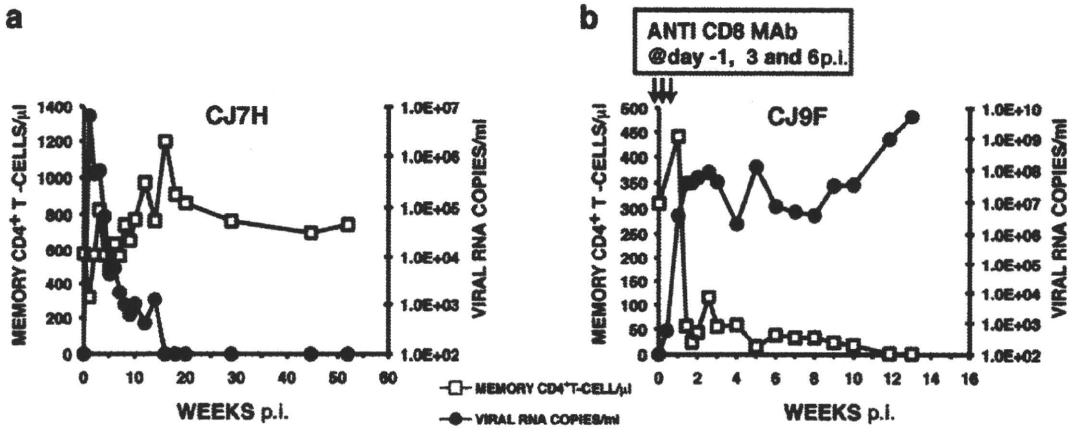


FIG. 1. Infectivity of the original $SHIV_{AD8}$ in rhesus monkeys. Macaques CJ7H (a) and CJ9F (b) were inoculated with 1 ml of undiluted $SHIV_{AD8}$ by the i.v., i.p., and BM routes. Macaque CJ9F was treated with the depleting anti-CD8 MAb cM-T807 as indicated.

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 increasingly 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

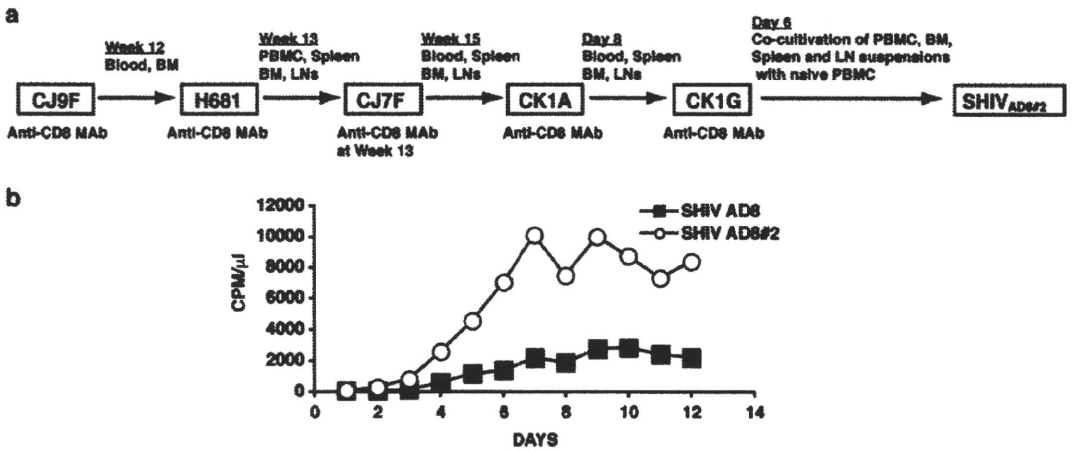


FIG. 2. Serial animal-to-animal passage of $SHIV_{AD8}$. (a) Passage history of $SHIV_{AD8}$ and origin of $SHIV_{AD8\#2}$. (b) Rhesus monkey PBMC were infected with $SHIV_{AD8}$ or the passaged $SHIV_{AD8\#2}$ virus stock, normalized for RT activity. CPM, counts per minute.

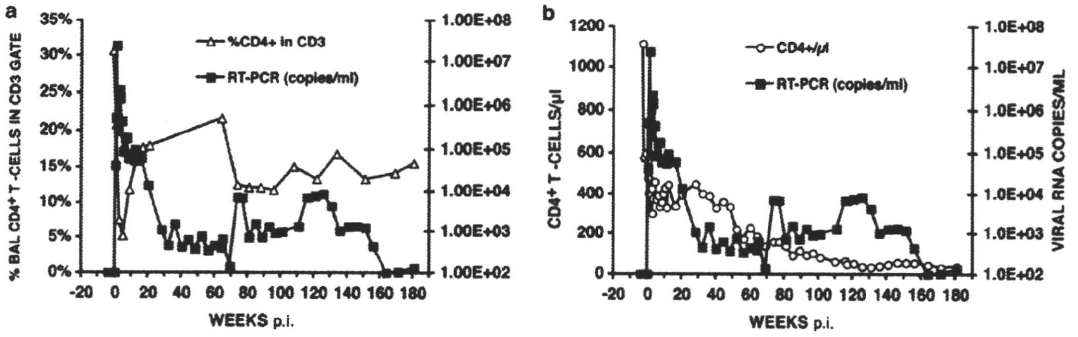


FIG. 3. $SHIV_{AD8\#2}$ induces sustained plasma viremia and loss of CD4 T cells in an inoculated rhesus macaque. Plasma viremia and the percentage of BAL fluid $CD4^+$ T cells (a) or the absolute numbers of circulating $CD4^+$ T cells (b) in rhesus macaque CJ8B inoculated intravenously with $SHIV_{AD8\#2}$ 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 $TCID_{50}$ 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 ($SHIV_{AD8PBMC}$) 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

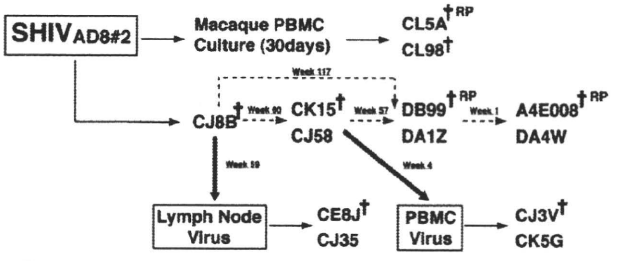


FIG. 4. $SHIV_{AD8\#2}$ and its immediate derivatives cause immunodeficiency in rhesus macaques. The dashed arrows indicate virus transfer by blood transfusion. The thick arrows indicate LN or PBMC specimens used to generate virus stocks by coculturing with PBMC from uninfected donors. †, euthanized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

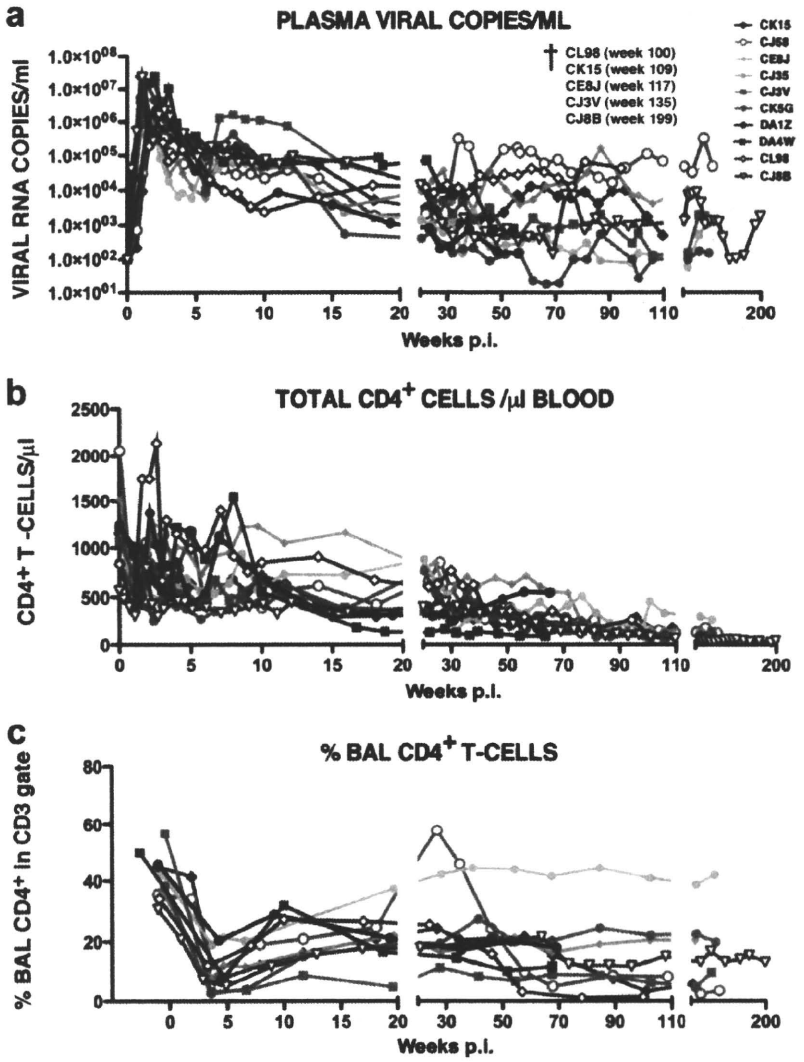


FIG. 5. Total $CD4^+$ T lymphocytes are gradually lost in normal progressors following infection with $SHIV_{AD8\#2}$ and its immediate derivatives. The levels of plasma viremia (a), absolute numbers of peripheral $CD4^+$ T cells (b), and percentages of BAL fluid $CD4^+$ T cells (c) are shown. The five 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 responses 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 $SHIV_{AD8\#2PBMC}$). Plasma neutralizing activity directed against the same virus used for animal challenge was evaluated in monkeys CJ8B, CE8J, CJ35, CJ3V, and

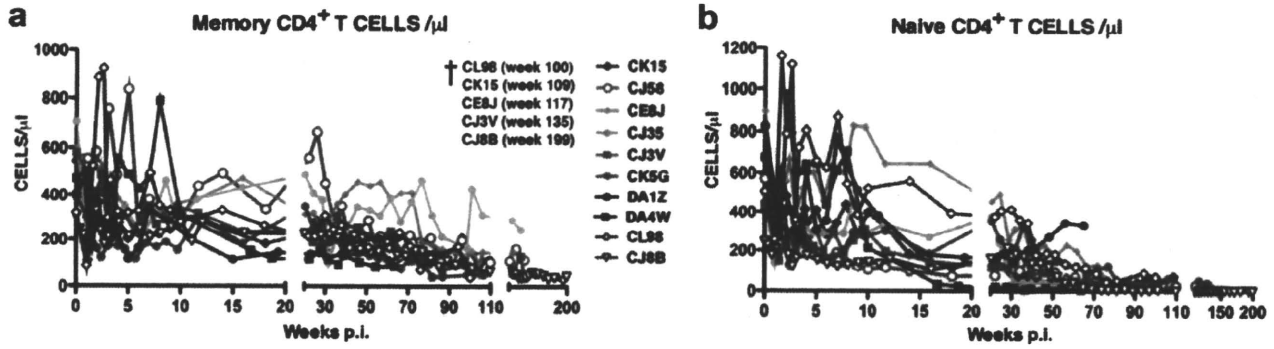


FIG. 6. Marked depletion of naïve and memory CD4$^+$ T lymphocytes characterizes long-term SHIV$_{AD8}$ infection in NP rhesus monkeys. Absolute numbers of memory CD4$^+$ T cells (a) and naive CD4$^+$ T cells (b) in 10 normal-progressor macaques during 200 weeks of SHIV$_{AD8}$ infection are shown. †,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

CK5G. The neutralization sensitivity of autologous virus (SHIV$_{AD8\#2PBMC}$) 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$_{AD8}$ NAbs, the actual 50% inhibitory concentration (IC$_{50}$) 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 naïve 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 naïve 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 naïve 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$_{AD8\text{-}DB99}$ required both inhibitors, whereas SHIV$_{AD8\text{-}A4E008}$ was inhibited only by the CCR5 inhibitor. This result indicates that SHIV$_{AD8\text{-}DB99}$ had acquired the capacity to use CXCR4 during its infection of macaque DB99 and that SHIV$_{AD8\text{-}A4E008}$ 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 naïve 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

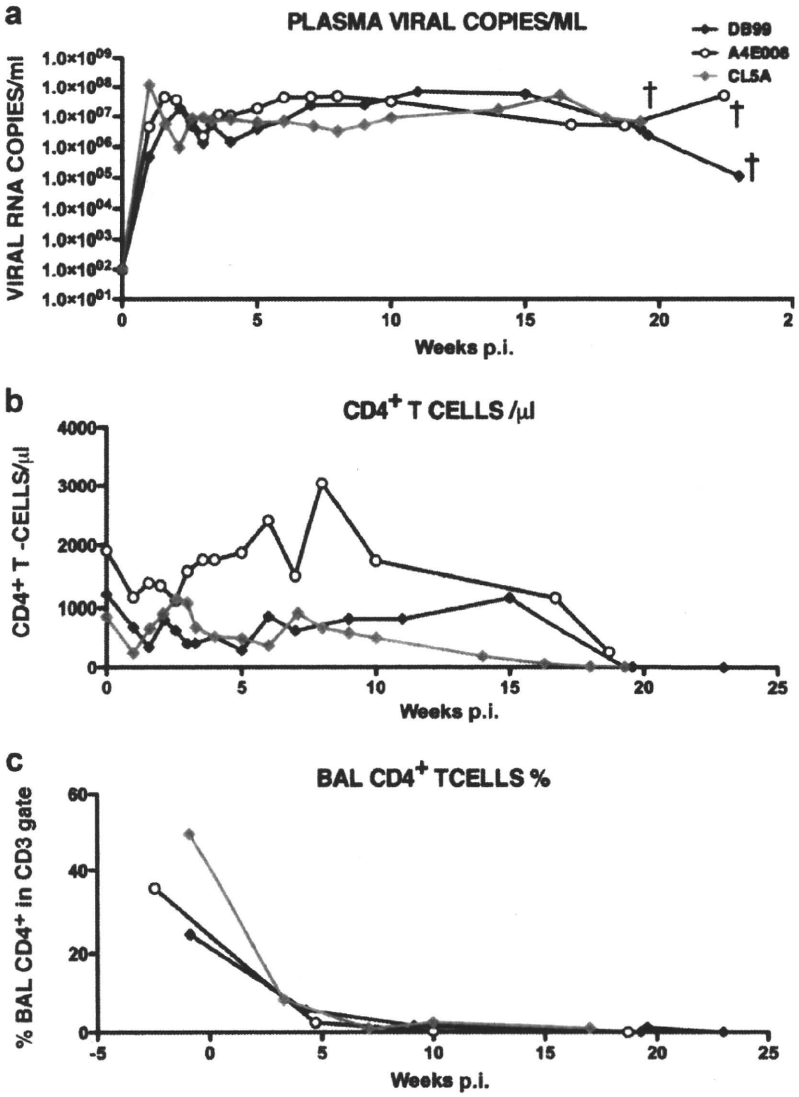


FIG. 7. Patterns of virus replication and $CD4^+$T cell dynamics in $SHIV_{AD8}$ rapid progressors. The levels of plasma viremia (a), absolute numbers of peripheral $CD4^+$ T cells (b), and percentages of BAL fluid $CD4^+$ T cells (c) are shown. †, euthanized animals.

$>1 \times 10^7$ 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

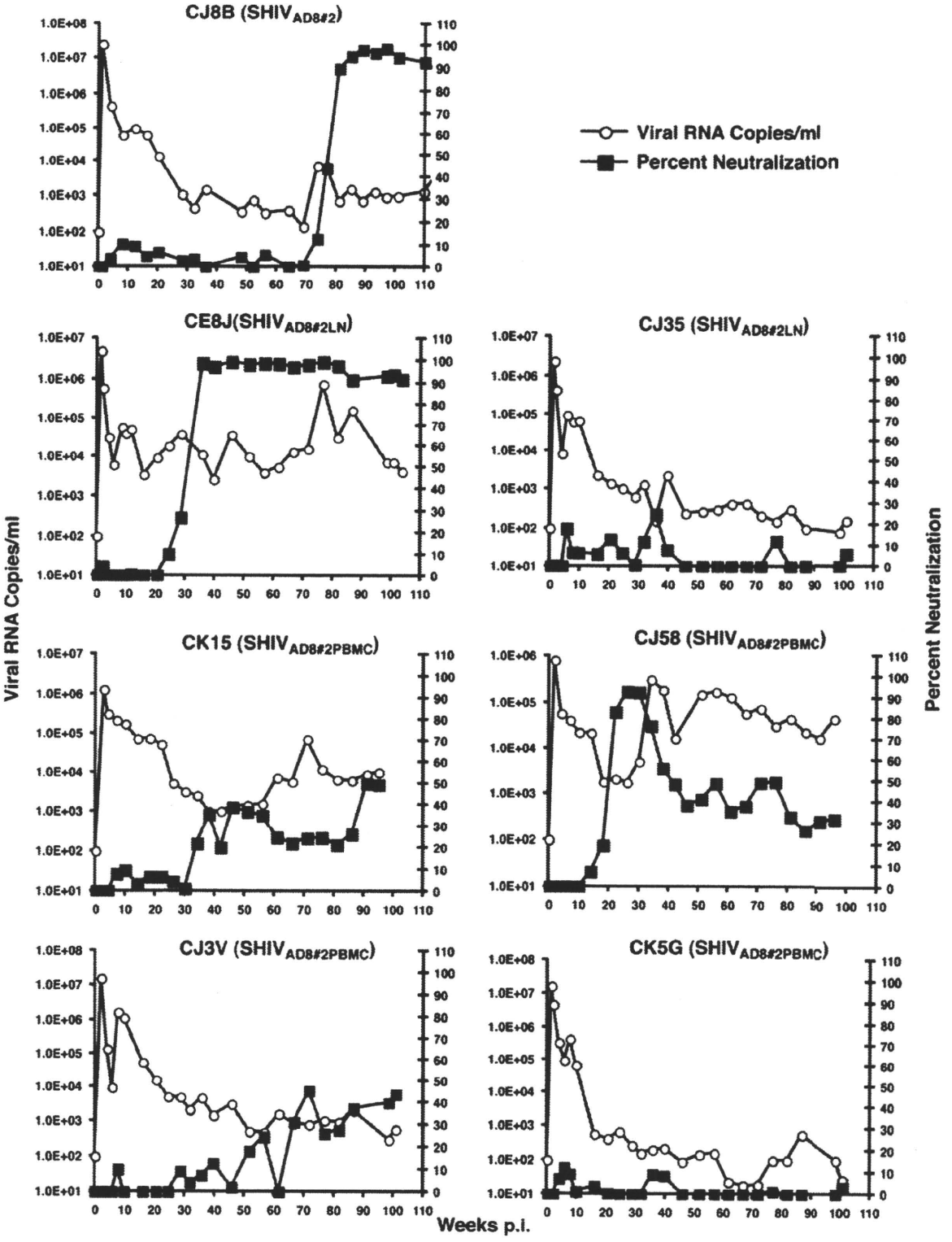
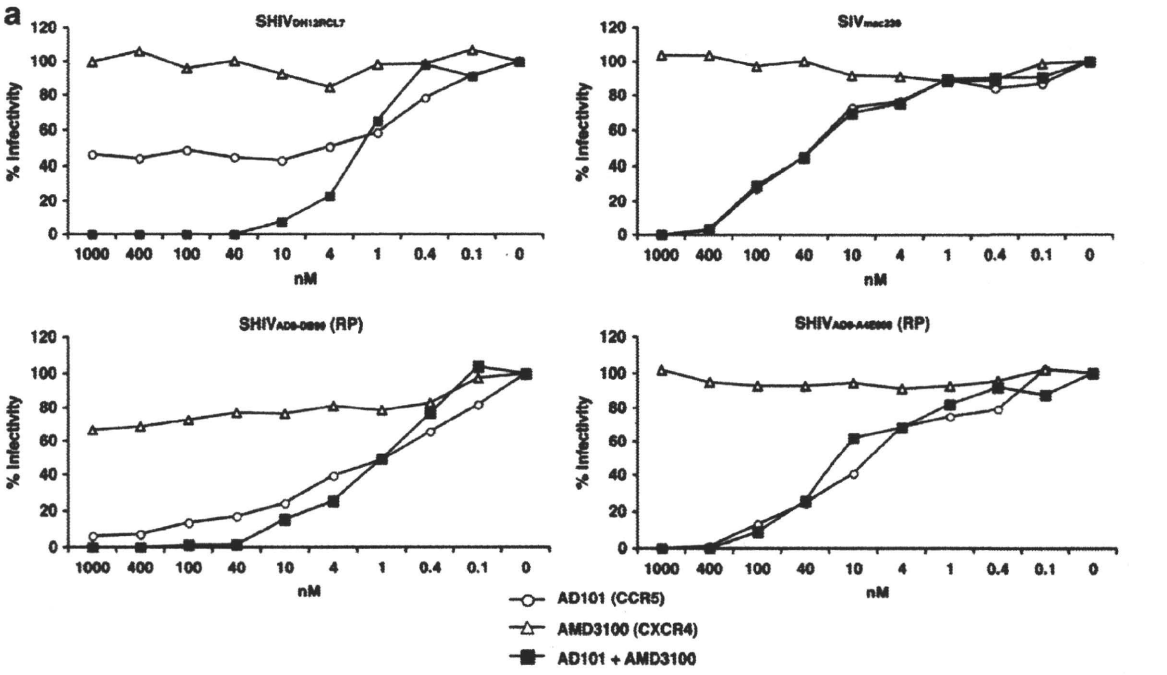


FIG. 8. Neutralizing-antibody activities detected in normal-progressor macaques following infection with $SHIV_{AD8\#2}$ or its immediate derivatives. Plasma samples (1:20 dilution) from the indicated $SHIV_{AD8}$-infected macaques were incubated in quadruplicate for 1 h at 37°C with the virus isolates 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.



b

VIRUS		N-TERMINAL V3 STEM	CORECEPTOR USAGE
$SHIV_{AD8}$		NNTRKSI---HI--GPGR	CCR5
$SHIV_{AD8\text{-}DB99}$	Rapid Progressor	NNTRKSIRIGHI--GPGR	CXCR4/CCR5
$SHIV_{AD8\text{-}A4E008}$	Rapid Progressor	NNTRKSIMIGHI--GPGR	CCR5
$SHIV_{AD8\text{-}CL5A}$	Rapid Progressor	NNTRKSI---HI--GPGR	(Undetermined)
$SHIV_{162P3}$		NNTRKSI---TI--GPGR	CCR5
$SHIV_{162\text{-}BR24N}$	Rapid Progressor	NNTRKSI---TIHRGPGR	CXCR4

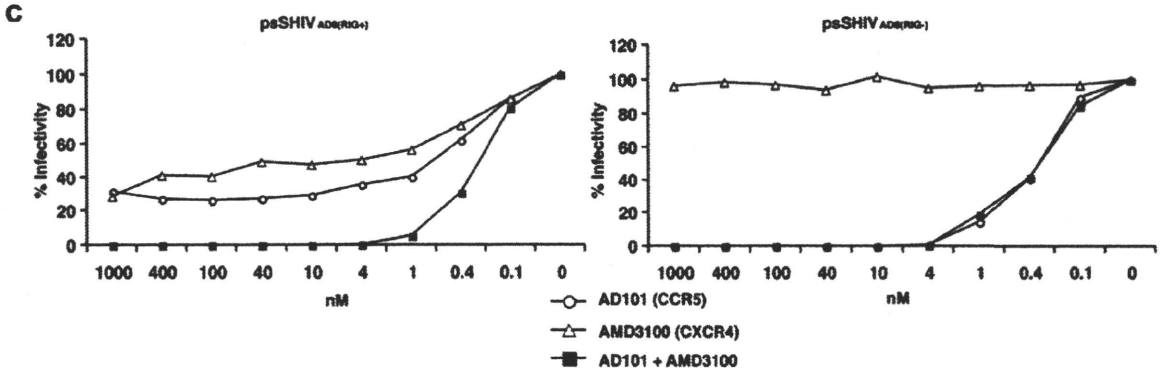


FIG. 9. Coreceptor utilization of $SHIV_{AD8}$ derivatives isolated from rapid progressors. (a) TZM-bl cells were infected in quadruplicate with viruses ($SHIV_{AD8\text{-}DB99}$ and $SHIV_{AD8\text{-}A4E008}$) recovered from rapid progressors DB99 and A4E008, respectively, in the presence of the indicated amounts of the small-molecule coreceptor inhibitors AD101 (CCR5), AMD 3100 (CXCR4), or both. SIV_{mac239} and $SHIV_{DH12RCL\text{-}7}$ 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 $SHIV_{SF162\text{-}BR24N}$ 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); *P. carinii* pneumonia
CJ58	Total $CD4^+$ T cells, 154/mm^3 (wk 111)
CE8J	Euthanized (wk 117); uncontrolled diarrhea, *C. coli* 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 *M. avium*

(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/μl, 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 differentiation or emigration of this T cell subset. It has also recently been reported that the loss of naive $CD4^+$ 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 $SHIV_{AD8\text{-}DB99}$, 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 $SHIV_{AD8\text{-}A4E008}$ 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

$SHIV_{AD8\#2LN}$, $SHIV_{AD8\#2PBMC}$, 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.

ACKNOWLEDGMENTS

We are indebted to Keith Reimann and the NIH Nonhuman Primate Reagent Resource for providing cM-T807; to the NIH AIDS Research and Reference Reagent Program for providing AMD3100; and to Julie Strizki, Schering-Plough, for providing AD101. We thank John Mascola for TZM-bl cells and instructions for performing virus neutralization assays, Robin Kruthers and Ranjini Iyengar for determining viral RNA levels, and Vanessa Hirsch for critical comments during the preparation of this paper. We appreciate the contributions of Boris Skopits in diligently assisting in the care and maintenance of our animals.

This work was supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

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