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Suppression of Virus Load by Highly Active Antiretroviral Therapy in Rhesus Macaques Infected with a Recombinant Simian Immunodeficiency Virus Containing Reverse Transcriptase from Human Immunodeficiency Virus Type 1

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We have modeled highly active antiretroviral therapy (HAART) for AIDS in rhesus macaques infected with a chimera (RT-SHIV) of simian immunodeficiency virus containing reverse transcriptase from human immunodeficiency virus type-1 (HIV-1). Seven RT-SHIV-infected macaques were treated with a combination of efavirenz (200 mg orally once daily), lamivudine (8 mg/kg subcutaneously once daily), and tenofovir (30 mg/kg subcutaneously once daily). Plasma viral RNA levels in all animals were reduced by more than 1,000-fold after 4 weeks and, in six of the seven animals, were reduced to undetectable levels after 10 weeks. Virus loads increased slightly between 12 and 16 weeks of treatment, associated with problems with the administration of efavirenz. After a change in the method of efavirenz administration, virus loads declined again and remained undetectable in the majority of animals for the duration of therapy. Treatment was stopped for three animals after 36 weeks of therapy, and virus loads increased rapidly. Posttreatment RT-SHIV isolates had no mutations associated with resistance to any of the three drugs. Efavirenz treatment was stopped, but lamivudine and tenofovir treatment for two other macaques was continued. The virus load in one of these two animals rebounded; virus from this animal was initially free of drug-resistance mutations but acquired the K65R mutation in reverse transcriptase at 11 weeks after efavirenz treatment was withdrawn. These results mimic HAART of HIV-1-infected humans. The RT-SHIV/rhesus macaque model should be useful for studies of tissue reservoirs and sites of residual replication that are not possible or practical with humans.

Highly active antiretroviral therapy (HAART) has been a significant advance in the treatment of AIDS. HAART has enabled the long-term suppression of human immunodeficiency virus type 1 (HIV-1) loads in many patients to low or undetectable levels, lowered mortality rates, and improved quality of life (8, 25). However, reservoirs of latent virus and residual viral replication persist (3, 25). Another major problem is the emergence of drug-resistant variants, which may lead to a rebound in virus load and treatment failure (7, 8, 19, 21, 25).

Three classes of antiretroviral drugs are widely used in various combinations for HAART: (i) nucleoside analog reverse transcriptase inhibitors (NRTIs), (ii) nonnucleoside reverse transcriptase inhibitors (NNRTIs), and (iii) protease inhibitors (PIs). Early HAART regimens included one PI and two NRTIs (13, 15). More recently, NNRTI use in HAART has increased due, at least in part, to toxicities associated with use of PIs. One of the most widely used NNRTIs is efavirenz (Sustiva). Efavirenz is used in HAART regimens that contain PIs and in PI-sparing regimens (14, 30). The combination of efavirenz with two NRTIs, lamivudine (3TC) and zidovudine, was shown

to more effectively lower plasma viral loads and delay the onset of virological failure and was better tolerated than a similar PI-based regimen (30).

In order to conduct more detailed studies of problems associated with viral resistance, persistence, and residual replication within the treated host, a suitable animal model for studies of HAART is needed. Simian immunodeficiency virus (SIV) infection of rhesus macaques has proven to be a useful animal model for studies of AIDS pathogenesis, but its usefulness as a model for HAART has been limited by the inability of commonly used NNRTIs to inhibit SIV replication. However, a chimera of SIV (RT-SHIV) in which the reverse transcriptase (RT) from SIVmac239 was replaced with the RT from an HIV-1 clone (HXBc2) is infectious for rhesus macaques (32) and susceptible to several nucleoside and nonnucleoside reverse transcriptase inhibitors (1, 2), including efavirenz (16), and to PIs (12). We report here the use of the RT-SHIV/rhesus macaque model to evaluate a HAART combination consisting of efavirenz (Sustiva), lamivudine [(−)-β-2′-deoxy-3′-thiacytidine], and tenofovir {9-[2-(phosphonomethoxy)propyl]adenine} (PMPA) (36).

MATERIALS AND METHODS

Virus and cells. Stocks of RT-SHIV and SIVmac239 were prepared from the appropriate 5′- and 3′-half clones, as described previously (12, 16). The 5′-half

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clone of the RT-SHIV containing the RT-encoding region from HIV-1 HXBc2 (32) was provided by Joseph Sodroski, Harvard Medical School, Boston, Mass. The 3′-half clone of SIVmac239, containing a *nef* open reading frame in the 3′-half clone, has been described previously (20). CEMx174 cells, which are permissible to both HIV-1 and SIV (26), were grown in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA) that was heat inactivated for 30 min at 56°C, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2.0 mM L-glutamine. All cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Infectious stocks of RT-SHIV were prepared following transfection of the two half clones into CEMx174 cells by electroporation, as previously described (22). Virus stocks were prepared from culture supernatants of infected CEMx174 cells. Cells were removed from the culture medium by centrifugation at $800 \times g$ for 10 min. Aliquots of supernatants were stored frozen at −130°C. The RT-SHIV stocks used for these studies had the T-to-C substitution at position 8 of the SIV tRNA primer binding site, which is necessary for rapid replication of RT-SHIV in vivo (29). These stocks were thawed and used for subsequent studies.

Antiviral drugs. Efavirenz was provided by Bristol-Myers Squibb, Wallingford, Conn., 3TC was provided by GlaxoSmithKline, Research Triangle Park, N.C., and PMPA was provided by Gilead Sciences, Foster City, Calif.

Animals and sample collection. Juvenile rhesus macaques (*Macaca mulatta*) 7 to 10 months old (~1.3 to 2 kg) were from the retrovirus-free colony of the California National Primate Research Center. This facility operates according to the Guide for the Care and Use of Laboratory Animals prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (22a). When necessary, animals were immobilized with ketamine-HCl (Parke-Davis, Morris Plains, New Jersey), 10 mg/kg body weight, injected intramuscularly. EDTA-anticoagulated blood samples were collected regularly to measure viral and immunologic parameters. Complete blood counts from these samples were done on an automated electronic cell counter (Baker 9000; Serano Baker Diagnostics), and differential counts were determined manually.

Virus inoculation. Animals were inoculated intravenously with 1.0 ml of virus containing 10^5 50% tissue culture infectious doses of cell-free RT-SHIV grown in CEMx174 cells.

Preparation and administration of drug. Efavirenz was fed at approximately 60 mg/kg per day by mixing the contents of a 200-mg Sustiva capsule (Bristol-Myers Squibb) into a peanut butter or peanut butter and jelly sandwich. Stock solutions of 3TC (16 mg/ml) were prepared in phosphate-buffered saline (pH 7.4). PMPA was suspended in distilled water (60 mg/ml) with NaOH added to a final pH of 7.0. Both 3TC and PMPA stocks were filter sterilized (pore size, 0.2 μm; Nalgene, Rochester, N.Y.) and stored at 4°C. These NRTIs were administered subcutaneously in the back at a regimen of 8 mg per kg body weight once daily for 3TC and 30 mg per kg body weight once daily for PMPA. Drug dosages were adjusted weekly according to body weight. The dose of PMPA was reduced to 15 mg/kg per day after 20 weeks of treatment to reduce any long-term renal toxicity (33). Serum chemistry panels and clinical and histopathological observations did not suggest any detectable toxicity for the efavirenz and 3TC regimens used in this study.

Virus isolation (cell free and cell associated). Cell-free and cell-associated infectious viruses were isolated by cocultivation of plasma or peripheral blood mononuclear cells with CEMx174 cells in 25-cm² flasks. SIV p27 core antigen production was measured by previously described methods (22, 34). Virus-positive cultures were centrifuged twice at $600 \times g$ for 5 min to remove cells. Aliquots of cell-free supernatants were stored at −80°C as virus stocks, and pellets of infected CEMx174 cells were resuspended in phosphate-buffered saline and also stored at −80°C. These isolates were used for DNA sequence analyses.

Nucleic acid preparation and sequence analysis. Total cellular DNA containing proviral DNA was extracted from infected cells by use of a DNeasy tissue kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. DNA was eluted from the silica columns by use of 100 μl buffer AE (QIAGEN). A 2-μl aliquot of each DNA preparation was amplified by nested PCR using JumpStart REDTaq (Sigma-Aldrich, St. Louis, Missouri). In the first round of nested PCR with RT-SHIV, 0.4 μM concentrations (each) of primers 239-2675 and 239-4751 (R) were used, and in the second round, 0.4 μM concentrations (each) of primers 239-2786 and 239-4615 (R) were added to each reaction. All primers are listed in Table 1, and conditions for PCRs were as described previously (22, 34). Amplicons were purified by use of either a PCR purification kit (QIAGEN) or Microcon YM-50 centrifugal filter devices (Millipore, Bedford, Massachusetts). DNA sequence analyses were performed as previously described (22, 34) with sequencing primers for HIV-1 RT listed in Table 1.

Viral RNA for RT-PCR was prepared from 140 μl of EDTA-anticoagulated,

TABLE 1. Primers used for PCR and DNA sequence analyses

Primer	3′ base	Primer sequence
SIV primers[a]		
239-2571	2571	CTGCACCTCAATTCTCTCTTTG
239-2675	2675	GTAACAGGAATAGAGTTAGGTCCAC
239-2786	2786	ATTAAAGGGACAATCATGACAG
239-4615 (R)	4615	TCTGTCTGGCCACTATTCTG
239-4751 (R)	4751	TCCACTAGCTACATGTACTGCAAC
HIV-1 primers[b]		
HXB2-2441	2441	GTAAGACAGTATGATCAGATACTC
HXB2-2493	2493	TACAGTATTAGTAGGACCTACAC
HXB2-EcoRV	2987	ACCAGGGATTAGATATCAGTAC
HXB2-3145	3145	TTAGAAATAGGGCAGCATAG
HXB2-3509	3509	CCAGTACATGGAGTGTATTATGAC
HXB2-3837	3837	GTGAAATTATGGTACCAGTTAG
HXB2-3039 (R)	3039	GGCTCTAAGATTTTTGTCAT
HXB2-3253 (R)	3253	GTACTGTCCATTTATCAGGAT
HXB2-4277 (R)	4277	AGCCATTGCTCTCCAATTACTG
HXB2-4384 (R)	4384	CTGGACTACAGTCTACTTGTC

[a] SIV primers are based on the sequence of SIVmac239 (GenBank accession number M33262). Nucleotides of SIV are numbered beginning from the 5′ end of the SIV genome (i.e., position 1 of SIV corresponds to nucleotide 257 of the M33262 sequence).
[b] GenBank accession number K03455.

cell-free plasma by use of a viral RNA kit (QIAGEN) according to the manufacturer's instructions. RNA was eluted from the silica columns in 50 μl of nuclease-free water. Nested RT-PCR was carried out under conditions previously described (22). Synthesis of cDNA was carried out using 5 to 20 μl of viral RNA. The first round of nested PCR was performed with primers 239-2675 and HXB2-3253 (R) or 239-2571 and 239-4751 (R), and the second round was performed with primers 239-2786 and HXB2-3253 (R) or 239-2675 and 239-4615 (R).

Plasma viral RNA levels. A real-time quantitative RT-PCR (TaqMan) assay with a sensitivity of 50 copies of viral RNA per ml of plasma was used to quantitate RT-SHIV RNA (17).

Lymphocyte phenotyping by four-color flow cytometry. T-lymphocyte antigens were detected by direct labeling of whole blood with peridinin chlorophyll protein-conjugated anti-human CD8 (clone SK1; Becton Dickinson Immunocytometry Inc., San Jose, CA), phycoerythrin-conjugated anti-human CD4 (clone M-T477; Pharmingen), fluorescein-conjugated anti-human CD3 (clone SP34; Pharmingen), and allophycocyanin-conjugated anti-human CD20 (clone L27; Becton Dickinson). Red blood cells were lysed, and samples were fixed in paraformaldehyde by using the Coulter Q-prep system (Coulter Corporation, Hialeah, Florida). Lymphocytes were gated by forward and side light scatter and were then analyzed with a FACSCalibur flow cytometer (Becton Dickinson). $CD4^+$ and $CD8^+$ T lymphocytes were defined as $CD3^+CD4^+$ and $CD3^+CD8^+$, respectively. B lymphocytes were $CD3^-CD20^+$.

RESULTS

Efficacy of efavirenz-based HAART. Two groups (A and B) of seven juvenile rhesus macaques each were used to study HAART in RT-SHIV infection. Group A animals were untreated, while group B received HAART. All animals became persistently infected after inoculation with RT-SHIV, and peak viral loads were reached between 2 and 4 weeks postinfection, as shown in Fig. 1. There was considerable variability in the virus loads of untreated group A animals (Fig. 1A). Four of the seven animals (33731, 33810, 33917, and 33741) maintained virus loads of 10^5 to 10^7 copies of RNA per ml of plasma for the duration of the experiment. Two group A macaques (33704 and 33753) had virus loads that declined to less than 10^4 copies of RNA per ml of plasma by week 10 and remained
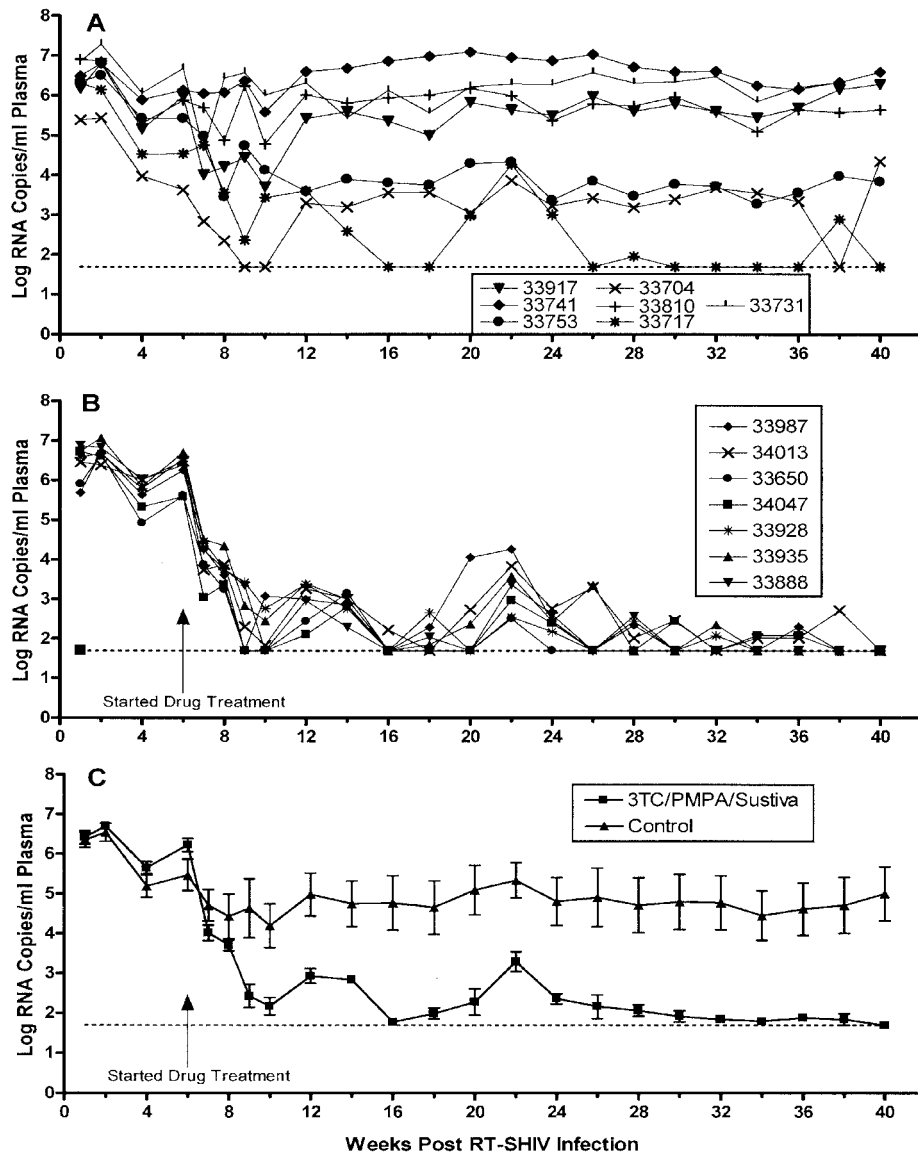
FIG. 1. Plasma viral RNA levels in RT-SHIV-infected macaques. (A) Control animals (no drugs). (B) Animals treated with efavirenz, 3TC, and PMPA. (C) Group averages of mean log values ± standard errors. Dashed lines represent the lower limit of detection of the assay (50 copies of viral RNA per ml of plasma). Arrows indicate the time that drug treatment was started.

between 10^3 and 2×10^4 copies per ml for the duration of the experiment. For one of these animals (33704), viral RNA was undetectable at weeks 9 and 10 but was detectable from week 12 onward. The virus load declined to undetectable levels at weeks 16 to 18 for one group A animal, with a few transient rebounds to 10^3 to 2×10^4 copies per ml afterwards (Fig. 1A).

Group B animals were treated with a combination of efavirenz (200 mg orally once daily), 3TC (8 mg/kg subcutaneously once daily), and PMPA (30 mg/kg subcutaneously once daily) beginning at 6 weeks postinfection. Viral RNA levels in all of the treated animals were reduced by more than 1,000-fold after 4 weeks of therapy (Fig. 1B). Viral RNA in three animals was below the level of detection (less than 50 copies per ml of plasma) at 3 or 4 weeks after initiation of therapy. Viral RNA in six of the seven drug-treated macaques was

undetectable at week 16 postinfection (10 weeks of therapy) (Fig. 1B). In contrast to the variability of virus loads in control (group A) animals, the patterns of virus load decline were very similar for all of the treated (group B) animals. This pattern during the first 10 weeks of therapy was similar to the biphasic decline in HIV-1 loads observed with HAART for human patients (3, 23)

Virus loads increased between weeks 18 and 22. This was associated with a compliance problem with several of the monkeys in the treated group that were not eating all of their efavirenz-containing food. Following adjustments in the feeding of the monkeys, plasma viral RNA levels declined and were undetectable in the majority of animals at each time sampled after 28 weeks postinfection (22 weeks after the initiation of therapy). Plasma viral RNA in all seven treated macaques was
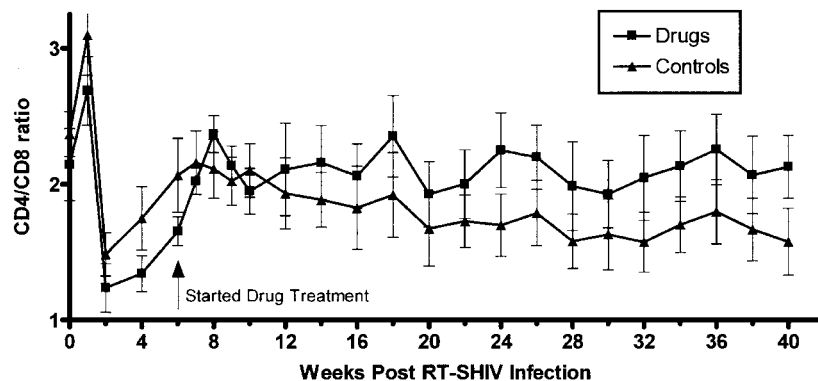
FIG. 2. Group averages of CD4$^+$/CD8$^+$ T-lymphocyte ratios in peripheral blood from RT-SHIV-infected macaques. Values are the means $\pm$ standard errors of the control group (no drugs) and the drug-treated group. The arrow indicates the time that drug treatment was started.

undetectable at week 40. The differences in plasma viral RNA levels of the control and drug-treated groups were statistically significant at all times evaluated between weeks 10 and 40 (P was <0.01 at all times except week 14, at which P was 0.015; Student's t test).

Absolute lymphocyte counts in peripheral blood were quite variable over time, as were absolute levels of CD4$^+$ and CD8$^+$ cell counts (data not shown). There was no significant difference between drug-treated and untreated groups in the percentages of CD4$^+$ T lymphocytes between weeks 7 and 40 of infection. However, drug-treated animals had significantly lower levels of CD8$^+$ T lymphocytes (data not shown) (P = 0.002; two-way analysis of variance) and significantly higher CD4/CD8 T-lymphocyte ratios (P < 0.0001; two-way analysis of variance) than the untreated animals for the period of treatment (week 7 to 42 of infection) (Fig. 2).

Drug treatment was stopped for three animals (33650, 33935, and 33987) from the treated group at week 42 (36 weeks of therapy). Treatment was changed to 3TC plus PMPA (no efavirenz) for two animals (33888 and 34047), and necropsies were performed on two animals from the treated group and three from the control (no drugs) group. Virus loads increased rapidly to levels of 6,000 to 140,000 copies/ml of plasma in all

three animals following the cessation of treatment (Fig. 3). DNA sequence analyses of the RT-encoding regions of viral isolates from these three animals revealed no mutations associated with resistance to any of the three drugs (Table 2). The only mutations detected in isolates from these three animals were G196R, which was present in isolates from all three macaques, K275R (animals 33935 and 33987), and L452S (animal 33650). We have previously reported the emergence of G196R and K275R in RT-SHIV from untreated macaques (16).

The virus load also rebounded for one animal (33888) after the removal of efavirenz from the drug regimen. By 3 weeks after the removal of efavirenz, G196R and K530R mutations were detected in the RT-encoding region of virus from this animal. Virus isolates obtained from this animal at 10 weeks after efavirenz removal had the K65R mutation in RT, which is associated with resistance to PMPA and low-level resistance to 3TC, as well as the G196R mutation. The K530R mutation in RT was no longer present at that time. Virus was undetectable, except for a transient virus flare at week 45, in the second animal (34047) (Fig. 3).

Similar to observations from previous studies (18), gross pathology and histopathology examination revealed changes
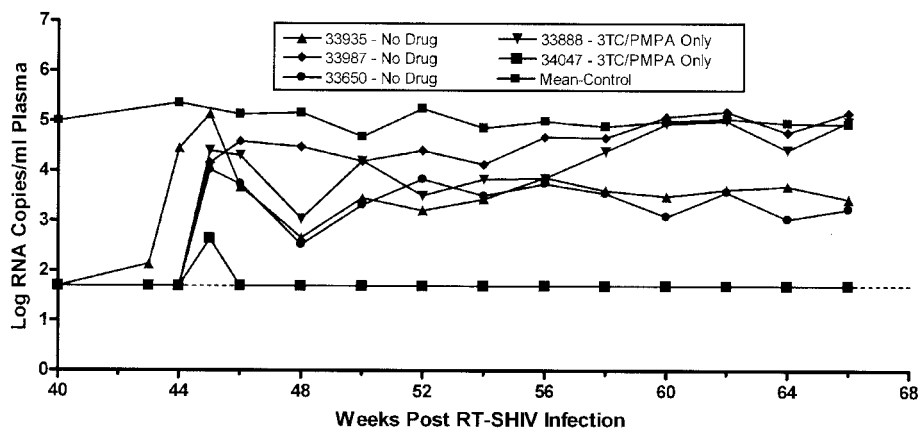

FIG. 3. Plasma viral RNA levels after alteration of drug therapy in RT-SHIV-infected macaques. At week 42, drug treatments were stopped for animals 33650, 33935, and 33987. Efavirenz treatment was stopped but treatment with 3TC plus PMPA continued for animals 33888 and 34047. For comparison, the mean values of control (group A) animals are also shown. The dashed line represents the lower limit of detection of the assay (50 copies of viral RNA per ml of plasma).

TABLE 2. Mutations in the RT-encoding regions of RT-SHIV
isolates from animals following drug treatment alterations

Treatment alteration	Animal	RT mutation(s) by wk[a]	
		45	52
No drug	33650	**	G196R, L452S
	33935	G196R, K275R	G196R
	33987	None	G196R, K275R
3TC + PMPA	33047	**	**
	33888	G196R, K530R	K65R, G196R

[a] **, virus or RT-PCR product was unable to be isolated for sequence analysis.

consistent with the levels of virus replication; the untreated animals with high viremia levels had pronounced generalized lymphoproliferative changes, with evidence of localized lymphoid depletion and early opportunistic infections (data not shown). In contrast, animals with low or undetectable viremia levels (either drug-treated or untreated) had mild or moderate lymphoproliferative changes, without signs of concurrent infections.

DISCUSSION

It has previously been shown that RT-SHIV can be useful for studies of NNRTIs (1, 2, 16). The results of this study demonstrate that RT-SHIV-infected rhesus macaques can be used for studies of HAART with drug combinations that include an NNRTI. This drug regimen consisted of an NNRTI (efavirenz) in combination with two NRTIs (3TC and PMPA). These drugs are approved by the Food and Drug Administration and widely used for HIV-infected humans. This combination reduced virus loads in RT-SHIV-infected macaques to very low or undetectable levels with kinetics similar to the biphasic decrease in virus loads produced by HAART regimens in HIV-1-infected humans (3, 23). Viral loads rebounded when this three-drug combination was terminated, as in HIV-infected humans, and virus isolates from these animals had none of the mutations known to confer resistance to any of these three drugs. Moreover, virus loads in the majority of control (no drug) animals were in the range of 10^5 to 10^7 copies of viral RNA per ml of plasma, again, similar to those in HIV-infected people. These similarities make RT-SHIV infection of rhesus macaques a highly relevant model for studies of HAART.

An advantage of the RT-SHIV model is that it can be used for studies of the three major classes of drugs that are widely used in AIDS therapy. SIV infection of rhesus macaques has provided an important animal model for studies of AIDS pathogenesis, but its use in studies of AIDS therapy has been mostly limited to studies of nucleoside analog inhibitors of RT (31, 34–36; reviewed in reference 11). SIV is similar to HIV-1 in its susceptibility to several of the protease inhibitors used in AIDS therapy (12), but there is little data on the efficacy of PIs in SIV-infected macaques. Unlike HIV-1 and RT-SHIV, SIV is not inhibited by NNRTIs. Therefore, most attempts to model HAART with SIV-infected macaques have used combinations of two or three NRTIs. The RT-SHIV/macaque model enables studies with NRTIs and NNRTIs, and it should also be useful for some studies with HAART combinations

that include PIs. However, mutants of SIV selected in vitro for resistance to protease inhibitors are genotypically different from HIV-1 mutants selected under the same conditions (A. C. Giuffre and T. W. North, unpublished data), so the RT-SHIV and SIV models may not be appropriate for studies involving resistance to PIs.

HAART for humans is currently limited by the inability of antiretroviral drugs to eliminate reservoirs of persistent virus. The most characterized reservoir of HIV-1 is the latently infected resting memory $CD4^+$ T cell (4–6, 9, 10, 23, 24, 37). Memory T cells have stably integrated provirus but require activation to produce infectious virus. This latent form is not susceptible to current antiviral drugs. Moreover, this pool of latently infected memory T cells is very stable, with a half-life of nearly 4 years (9). Latently infected memory T cells in peripheral blood and lymph nodes of HIV-1-infected individuals have been detected, and they are also likely to exist in the gastrointestinal tract and other lymphoid tissues. These reservoirs of latent virus represent a major barrier to the eradication of HIV-1 with antiretroviral therapy (28). And there are likely to be other cellular and tissue reservoirs of HIV-1.

Like HIV-1, reservoirs of latent SIV have been detected in resting memory T-cells from peripheral blood, lymph nodes and spleen, but not thymus (27). It is likely that latent RT-SHIV also persists in these cells. RT-SHIV isolates we obtained after withdrawal of drugs had no drug-resistance mutations; this is consistent with reactivation of RT-SHIV from memory T cells or other reservoirs of latency that were established early in infection, prior to the onset of therapy. Accordingly, the RT-SHIV/model should be valuable for detailed investigation of latency during HAART and reactivation of latent virus upon cessation of therapy.

A successful nonhuman primate model of HAART provides the opportunity to better study tissue reservoirs of latent virus and sites of residual virus replication during effective drug therapy. Although resting memory $CD4^+$ T lymphocytes are known to be reservoirs of latent HIV-1 in blood and peripheral lymph nodes of infected individuals (4–6), it has not been determined whether these reservoirs also reside in other lymphoid tissues, such as thymus and spleen. It is also likely that there are other reservoirs of latent HIV-1 that remain to be identified. These may include macrophages, follicular dendritic cells, and anatomical sanctuaries, such as the central nervous system and genitourinary tract (3). These reservoirs of latent virus are particularly problematic because they are not susceptible to the replication inhibitors used in HAART, and latent virus can reactivate when therapy is stopped. In addition, there is evidence of low level virus replication (residual replication) during HAART even in patients that have virus loads below the level of detection (25). The RT-SHIV/macaque model that we have described will permit invasive studies to more fully define sites of latency and of residual virus replication during HAART. The model will also be useful to test novel or risky strategies to eliminate latency and/or residual virus replication that may not be feasible to test in humans.

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