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Suppression of Viremia and Evolution of Human Immunodeficiency Virus Type 1 Drug Resistance in a Macaque Model for Antiretroviral Therapy▽

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Antiretroviral therapy (ART) in human immunodeficiency virus type 1 (HIV-1)-infected patients does not clear the infection and can select for drug resistance over time. Not only is drug-resistant HIV-1 a concern for infected individuals on continual therapy, but it is an emerging problem in resource-limited settings where, in efforts to stem mother-to-child-transmission of HIV-1, transient nonnucleoside reverse transcriptase inhibitor (NNRTI) therapy given during labor can select for NNRTI resistance in both mother and child. Questions of HIV-1 persistence and drug resistance are highly amenable to exploration within animals models, where therapy manipulation is less constrained. We examined a pigtail macaque infection model responsive to anti-HIV-1 therapy to study the development of resistance. Pigtail macaques were infected with a pathogenic simian immunodeficiency virus encoding HIV-1 reverse transcriptase (RT-SHIV) to examine the impact of prior exposure to a NNRTI on subsequent ART comprised of a NNRTI and two nucleoside RT inhibitors. K103N resistance-conferring mutations in RT rapidly accumulated in 2/3 infected animals after NNRTI monotherapy and contributed to virologic failure during ART in 1/3 animals. By contrast, ART effectively suppressed RT-SHIV in 5/6 animals. These data indicate that suboptimal therapy facilitates HIV-1 drug resistance and suggest that this model can be used to investigate persisting viral reservoirs.

Human immunodeficiency virus type 1 (HIV-1), the major cause of AIDS, is the most widely disseminated, deadly infectious disease of our era. It is estimated that 40 million people worldwide were living with HIV-1 and approximately 3 million people died from AIDS in 2005 (29). In developed nations, access to antiretroviral therapy (ART) has decreased the number of AIDS-related deaths. While ART can suppress HIV-1 for a number of years in infected individuals, it does not eradicate the virus (25). When ART is removed, viremia typically rebounds quickly to pretherapy levels (8, 20, 26, 43). The ability of HIV-1 to persist during ART highlights the problem of virus production from long-lived, infected producer cells that current therapy and host immune responses are unable to eliminate (25). Understanding of what cells and tissues actively reseed infection after cessation of therapy has been hampered by limited animal models that can recapitulate suppressive ART during a pathogenic infection.

Another consequence of continual antiretroviral use has been the emergence of drug-resistant virus, creating a challenge for the treatment of HIV-infected individuals. Standard ART often consists of multiple drugs targeting HIV-1 reverse transcriptase (RT): two nucleoside RT inhibitors (NRTIs) and one nonnucleoside RT inhibitor (NNRTI) (45). Mutations within the RT coding region of HIV-1 can lead to resistance to multiple compounds, essentially rendering them ineffective. For example, mutations associated with resistance to a NNRTI often confer resistance to all drugs in that class. Recently it was reported that the prevalence of sexually transmitted viruses resistant to NRTIs or NNRTIs can be as high as 15 to 25% in populations with access to ART (23, 36, 54, 60).

It is hypothesized that many antiviral resistance-conferring mutations in HIV-1 exist prior to therapy, and this resistance is engendered by the large number of replication cycles achieved by HIV-1 during infection, which enhances viral diversity (10). For this reason, the success of ART has, in part, relied on the low probability of multiple resistance-conferring mutations ac-

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cumulating on one viral genome (53). Suboptimal therapy undermines this strategy by increasing the representation of viral genomes with single resistance-conferring mutations on which other amino acid substitutions can accumulate, thereby accelerating the development of multidrug-resistant forms of HIV-1.

In addition to horizontal transmission of antiviral resistance, mother-to-child transmission of drug-resistant HIV-1 has been an unintended consequence of efforts to curb vertical transmission of the virus. In resource-limited settings, where ART often is not available, pregnant HIV-infected women are given a single dose of the NNRTI nevirapine (NVP) at the onset of labor (4, 24, 28, 34, 41, 57). This inexpensive treatment of the mother and in some cases also the infant can significantly decrease mother-to-child transmission by as much as 50%. The long pharmacokinetic decay of NVP means limited dosing can provide effective antiviral drug levels through the peripartal period (40, 42). However, this extended half-life also means that once treatment is stopped, viral replication can occur in a setting of suboptimally suppressive drug concentrations, creating an ideal situation for the emergence of drug resistance. Indeed, selection by NVP of drug resistance-conferring mutations in the RT of the replicating plasma virus pool was observed in 15 to 50% of the mothers (11, 14, 16, 30, 35) and in 45 to 87% of the infants (15, 16, 38) as assessed by population-based sequencing. All of these studies found K103N to be the most prevalent amino acid substitution observed for women with NVP-resistant virus. Sensitive detection methods for NVP resistance-conferring mutations showed that more than 90% of women who received single-dose NVP still had detectable resistance-conferring mutations more than a year after dosing (18, 47). There is recent evidence that women treated with single-dose nevirapine are at higher risk of failure for NVP-containing triple therapy within 6 months (37).

In this study, we used a macaque model to study the effectiveness of a commonly used ART regimen and the impact of prior exposure to NNRTI monotherapy. Pigtail macaques were infected with a defined inoculum of simian immunodeficiency virus (SIV) containing HIV-1 RT (RT-SHIV$_{mne}$) (2). Unlike SIV, this chimeric virus is inhibited by all FDA-approved RT inhibitors. In a pilot study, RT-SHIV$_{mne}$ replicated efficiently in vivo and was pathogenic in macaques, enabling consideration of longer-term ART studies to evaluate persistence. In a separate group of animals, we studied the effects of short-course NNRTI monotherapy on development of drug resistance and the impact it might have on subsequent combination ART. Short-course NNRTI monotherapy proved effective in increasing the relative frequency of mutant viruses resistant to the drug. Weeks later, the application of ART revealed that prior monotherapy accelerated the emergence of multidrug-resistant virus in one animal, with a particular resistance combination rapidly predominating in the population. The dynamics of this mutant population were carefully followed. By contrast, in the animals that did not fail therapy, we found that ART suppressed RT-SHIV$_{mne}$ replication in vivo by up to 4 orders of magnitude within 5 to 6 weeks of treatment, strongly resembling HIV-1 decay in humans on therapy. Finally, by interruption of therapy in animals with suppressed viremia, we were able to demonstrate that RT-SHIV$_{mne}$ was still present in all animals and consisted of the wild type or single-drug-resistant variants.

MATERIALS AND METHODS

Virus. 293T cells were transfected with a plasmid encoding the RT-SHIV$_{mne}$ provirus using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). CEMx174 cells were infected with the transfection supernatant in RPMI 1640 medium (Invitrogen) containing 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.3 mg/ml L-glutamine (Invitrogen). RT-SHIV$_{mne}$ was passaged in CEMx174 cells until the titer reached 1×10^5 infectious units/ml as determined on TZM indicator cells, formerly called JC53 BL13+ cells (13).

Animals. Four pigtail macaques (*Macaca nemestrina*) were housed at the NIH (pilot group), and six pigtail macaques were housed at the Washington National Primate Research Center (ART group) in accordance with American Association for Accreditation of Laboratory Animal Care standards. All animals were negative for serum antibodies to HIV type 2, SIV, type D retrovirus, and simian T-lymphotropic virus type 1. Infections and blood draws were conducted while the animals were sedated with intramuscular injection of tiletamine-zolazepam (Telazol; 3 to 6 mg/kg; NIH) or ketamine (10 to 15 mg/kg; Washington National Primate Research Center). Blood was drawn into EDTA- or heparin-treated tubes at the time points indicated. Plasma was separated from the blood by centrifugation and was frozen at −80°C in aliquots.

Plasma viral loads and CD4 counts. Viral RNA (vRNA) was extracted from plasma taken at each time point for each animal, and quantitative RT-PCR was performed on the vRNA to determine the number of viral genomes per ml of plasma, as previously described (9). The assay limit of detection was 15 copies/ml plasma. Lymphocyte subsets (CD3, CD4, CD8, and CD20) were measured by staining whole blood and analyzed by flow cytometry.

RT inhibitors and dosing. Three doses of efavirenz (EFV) from 200-mg capsules (Sustiva; Bristol Myers-Squibb, Princeton, NJ) were mixed in yogurt and fed to three macaques in the ART group (M03250, M04007, and M04008) during week 13, days 1, 2, and 4. The other three animals in the ART group received no EFV at week 13. At week 17, all animals in the ART group were given daily doses of tenofovir disoproxil fumarate (TDF) (20 mg/kg) and emtricitabine (FTC) (50 mg/kg) subcutaneously (Gilead Sciences, Foster City, CA) and EFV (200 mg in various food treats) orally for up to 20 weeks (27, 52). Drug administration was discontinued for all animals for 2 to 4 weeks until plasma viral loads reached 3,000 copies/ml or higher. All three drugs were then restarted until 45 weeks postinfection (three to five additional weeks). EFV levels were measured in plasma samples by high-performance liquid chromatography (Infectious Disease Pharmacokinetics Laboratory, National Jewish Medical and Research Center, Denver, CO).

Measurement of drug resistance-conferring mutations. vRNA was extracted from the challenge virus stock and plasma samples from the ART-treated and untreated control group animals and cDNAs were synthesized as previously described (48). Allele-specific real-time PCR (ASP) was used to measure the frequencies of the codons encoding the following residues: K65, 65R, K103, 103N (AAC and AAT), Y181, 181C, M184, 184I, and 184V, as previously described (46). ASP was performed on samples having 3,000 vRNA copies or higher.

Single-genome sequencing (SGS) was performed as previously described (48). Briefly, cDNA was diluted to a single viral copy, and a fragment encompassing the first 600 nucleotides of RT was sequenced. Approximately 35 clones containing a single viral copy were sequenced for each sample and aligned using ClustalW. Genetic diversity was calculated as average pairwise differences using hmmr-1.8.5.

RESULTS

RT-SHIV$_{mne}$ pathogenesis. RT-SHIV$_{mne}$ was derived from RT-SHIV$_{mne\ cl.\ 8}$, which is SIV$_{mne\ cl.\ 8}$ with the RT coding region replaced with the HIV-1$_{HxB2}$ RT coding region (2). RT-SHIV$_{mne\ cl.\ 8}$ was additionally modified by replacing the 3′ half of the virus containing part of the *vif* gene (base 5243) through the 3′ long terminal repeat with that of SIV$_{mne027}$, a highly pathogenic and macrophage-tropic isolate derived from in vivo passaging of SIV$_{mne\ cl.\ 8}$ (31, 32). This newly derived virus is hereafter referred to as RT-SHIV$_{mne}$.

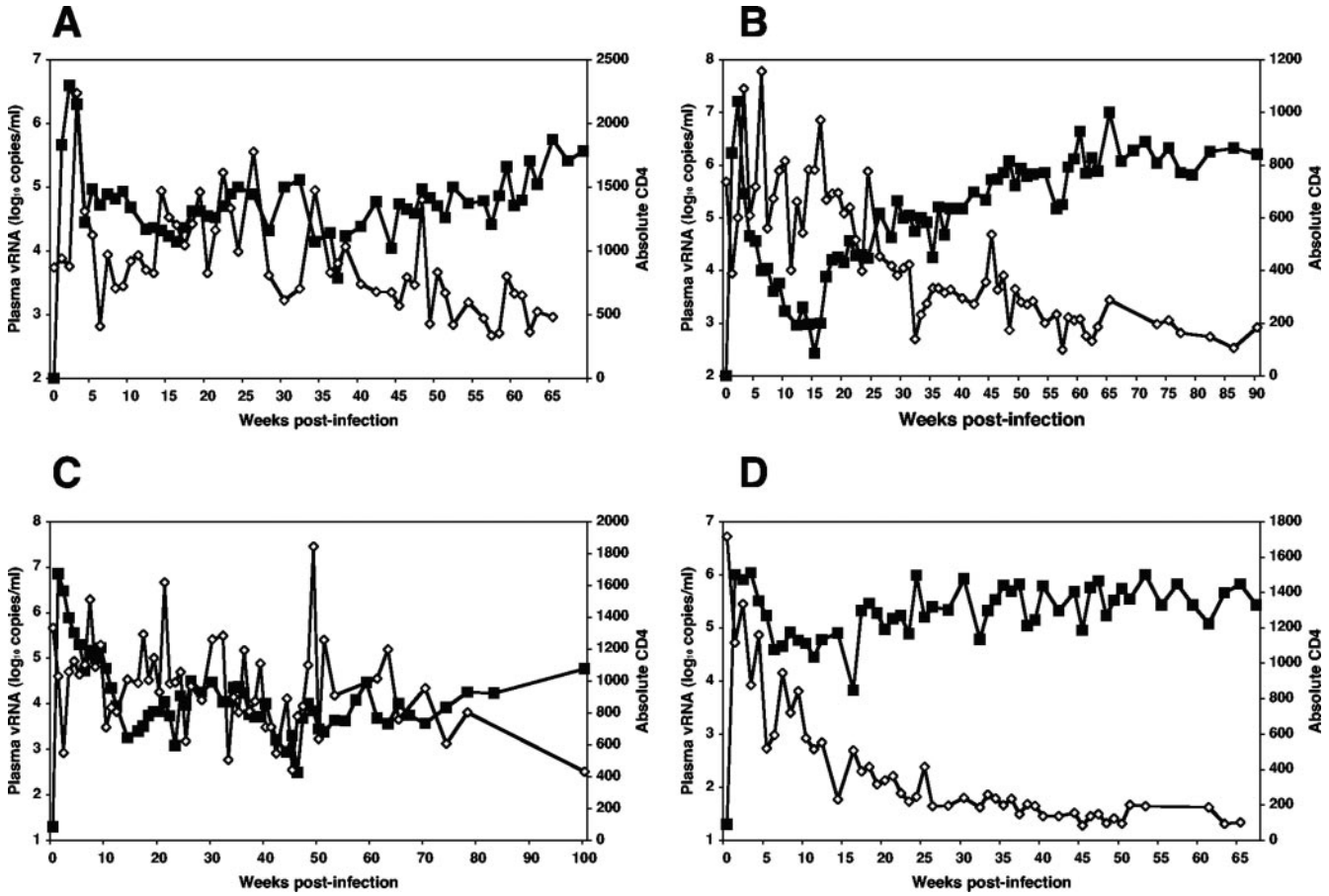

FIG. 1. Plasma viral loads versus CD4 counts for each animal in the pilot study. Four animals were challenged with RT-SHIV$_{mne}$ from cell culture supernatant at week 0, and plasma vRNA levels (filled symbols) and CD4$^+$ cell numbers (open symbols) were measured for animals 96P071 (A), 97P015 (B), 97P029 (C), and 96P084 (D).

A pilot study was initiated to evaluate the replication and pathogenesis of RT-SHIV$_{mne}$ in vivo. Four pigtail macaques were infected intravenously with 10^5 infectious units each of virus from cell culture supernatants. All animals showed persistent viral replication. Viral loads were closely monitored for the animals in the pilot group for up to 100 weeks postinfection (Fig. 1A to D). Initial plasma virus peaks were observed at 1 to 2 weeks postinfection, with levels of 1×10^6 to 1.6×10^7 vRNA copies/ml (average, 7.0×10^6 copies/ml). Plasma virus set points for these animals were variable and ranged between 10^3 and 10^5 vRNA copies/ml.

All animals showed pathogenesis and onset of disease, reflected by progressive declines in CD4$^+$ T cells. Peripheral CD4$^+$ T-cell counts were continuously monitored for the four pilot study animals (Fig. 1A to D). They ranged from 700 to 1,700 cells/μl prior to infection. Declines in CD4$^+$ T cells to below 500 were observed for all animals, with counts for 2 animals declining to less than 200 (Fig. 1B and 1D). Sustained high plasma viral loads or increases in viral loads for all animals were correlated with declines in peripheral CD4$^+$ T cells, indicative of pathogenic infection.

Three of the four pilot group animals were euthanized for untreatable symptoms associated with AIDS. These animals had moderate to severe weight loss and/or had respiratory and gastrointestinal pathologies. The only relatively healthy RT-SHIV$_{mne}$-infected animal, 96P029, had the lowest plasma viral load and the highest CD4$^+$ T-cell counts (Fig. 1C). This range of outcomes is typical of macaques infected with the parental SIV$_{mne027}$ (31).

The pilot group animals were also used to evaluate pharmacokinetics of NNRTIs. Single-dose NVP treatment (100 or 200 mg; oral solution or crushed pills) was initially tested with these animals at 16 weeks postinfection or later. There was no plasma NVP detected at 24 h or 48 h after drug administration and no discernible decline in plasma viremia in any of the animals (data not shown). Because of the apparent poor oral bioavailability and/or pharmacokinetics of NVP in pigtail macaques, we tested the NNRTI EFV in all animals. A single dose of oral EFV was administered at week 45 or 57 postinfection and was detected in the plasma up to 14 days, while viral loads dropped four- to eightfold in all animals at 72 h after dosing (data not shown).

Short-course EFV monotherapy rapidly selects for resistance. To study more fully the effects of brief NNRTI treatment on evolution of resistance and subsequent therapy, six new macaques were infected with RT-SHIV$_{mne}$. Thirteen weeks later, short-course EFV monotherapy (three doses in 4 days) was given to three of these animals. The other three were

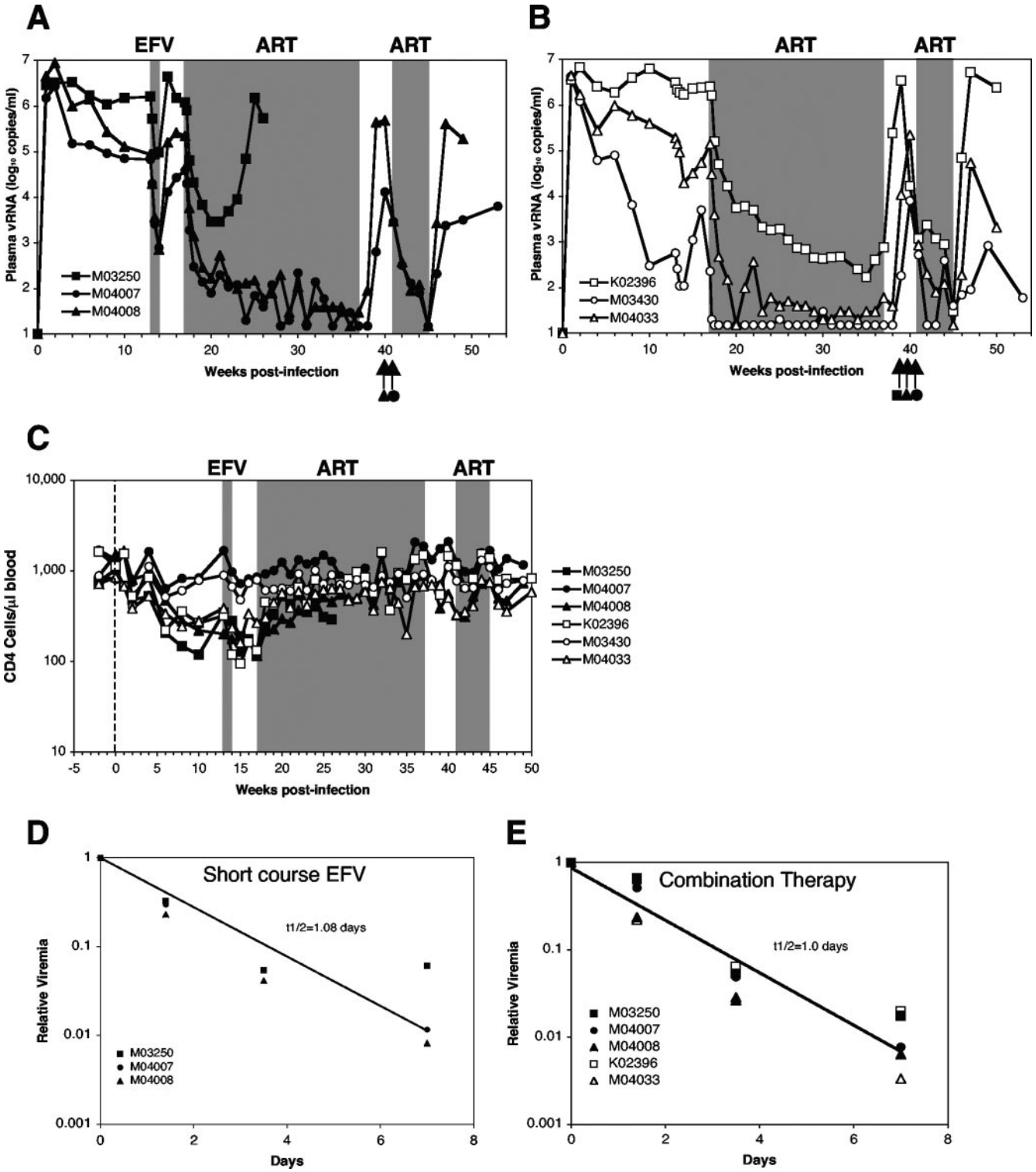

FIG. 2. Plasma viral loads and CD4 counts of animals receiving ART. Animals were challenged intravenously with 1×10^5 infectious units of RT-SHIV$_{mne}$ at week 0. (A) Three animals were given EFV monotherapy for 3 days during week 13. Daily ART was administered for 20 weeks (17 to 37 weeks postinfection). Animal M03250 was euthanized after virologic failure at week 26. ART was discontinued for the other two animals and reinitiated at week 40 or 41, continuing until week 45. (B) Three animals were given daily ART for 20 weeks (17 to 37 weeks postinfection). ART was discontinued and reinitiated at week 39, 40, or 41, continuing until week 45. (C) Absolute CD4 counts of the ART group animals were measured in the blood at multiple time points. Gray shading represents the time when animals received therapy. Arrows denote when ART was reinitiated for each animal after treatment interruption. The plasma virus decay kinetics are shown for the animals during the week of EFV monotherapy (week 13-14) (D) and the first week of combination therapy (week 17-18) (E). The viral loads are plotted relative to the last pretherapy value. Regression lines for all animals combined are shown for EFV monotherapy and combination therapy, along with the inferred half-lives (r^2 values are 0.83 and 0.91, respectively).

TABLE 1. Frequencies of resistance-conferring mutations in RT of RT-SHIV-infected animals before and after treatment as determined by ASP

Animal	Treatment group	Wk postinfection	Wk post-initiation of treatment		Frequency of mutation (%)[c]				
			EFV	ART	K65R	K103N		M184I	M184V
						AAC	AAT		
M03250	EFV monotherapy	13	0	0	—	—	—	—	—
		14	1	0	—	8	13		
		17	4	0	—	12	18	—	—
		23	NA[d]	6	18	24	1	3	—
		24	NA	7	5	60	0.3	24	0.5
		25	NA	8	3	94	0.07	71	7
		26	NA	9	1	100	—	88	6
M04007	EFV monotherapy	13	0	0	ND[e]	ND	ND	ND	ND
		17	1	0	—	—	—	—	—
		40	NA	23[a]	—	—	—	—	—
		49	NA	32[b]	—	—	—	—	—
M04008	EFV monotherapy	13	0	0	—	—	—	—	—
		15	1	0	—	18	21	—	—
		17	NA	0	—	8	16	—	—
		21	NA	4	—	15	10	—	—
		25	NA	8	—	20	15	—	—
		28	NA	11	—	14	21	—	—
		40	NA	23[a]	—	5	31	—	—
		47	NA	30[b]	—	27	40	—	—
K02396	No monotherapy	13	NA	0	ND	ND	ND	ND	ND
		17	NA	0	—	—	—	—	—
		39	NA	22[a]	—	—	—	—	—
		47	NA	30[b]	—	—	—	—	—
M03430	No monotherapy	13	NA	0	ND	ND	ND	ND	ND
		17	NA	0	—	—	—	—	—
		47	NA	30[b]	—	—	—	—	—
M04033	No monotherapy	13	NA	0	ND	ND	ND	ND	ND
		17	NA	0	—	—	—	—	—
		40	NA	23[a]	—	—	—	—	—
		47	NA	30[b]	—	—	—	—	—

[a] During treatment interruption.
[b] After discontinuation of ART.
[c] —, none detected; limit of detection, 0.01% for K103N AAC or K103N AAT, 0.3% for M184V, 0.05% for M184I, and 0.2% for K65R.
[d] NA, not applicable.
[e] ND, not done.

left untreated. Plasma viremia in the EFV-treated animals dropped 18-, 86-, and 123-fold within 7 days after the drug administration began (Fig. 2A), with plasma EFV levels of more than 50 ng/ml (158 nM) measured 4 to 7 days after dosing (data not shown). Despite a nearly 100-fold difference at initiation of EFV, viremia in all three animals displayed similar rates of decline, with a half-life of approximately 1 day (Fig. 2D). The untreated animals in the cohort had small (2 to 9-fold) decreases in plasma virus load during the same time period (Fig. 2B) and no detectable plasma EFV (data not shown).

With the EFV-treated animals, ASP analysis showed no common NNRTI-associated resistance mutations (K103N or Y181C), FTC-associated resistance mutations (M184I/V), or TDF-associated resistance mutation (K65R) prior to EFV monotherapy within the sensitivities of the respective assays. However, for 2/3 animals given short-course EFV, K103N amino acid substitutions (both AAC and AAT codons) were readily detectable within 4 weeks after the drug was administered (17 weeks postinfection) (Table 1). For the treatment-naive animals, K103N was not found above the assay background level. None of the animals had any detectable K65R or M184I/V substitutions at this point. No Y181C amino acid substitutions were detected by ASP at any time during the study.

Antiretroviral therapy effectiveness in treatment-naive and experienced animals. To evaluate the effect of a short-course EFV regimen on subsequent triple therapy, a combination of TDF, FTC, and EFV was administered daily to all six of the ART group animals for 20 weeks, beginning at 17 weeks postinfection. This combination is widely used for treatment of HIV-infected individuals (22).

Plasma viral loads declined significantly in all animals on ART (Fig. 2A and B), with an excellent correlation between the level of pretherapy viremia and the extent of decline. The animals pretreated with EFV had >400-fold declines in plasma viremia at their lowest measured levels. All of the five evaluable animals showed identical initial rates of decay, indepen-

dent of prior treatment or level of viremia, and indistinguishable from the decay observed following EFV monotherapy (Fig. 2E). Two of the animals had viral loads at or near the limit of detection of the assay (15 vRNA copies/ml) during the majority of the treatment period. One animal began to fail therapy after only 6 weeks of ART, with increasing viremia despite continued treatment. This animal had the highest viral load and had significant frequencies of K103N present in the viral population prior to combination therapy.

Two of the animals that had not received EFV monotherapy pretreatment prior to initiation of combination ART had on-therapy viral loads at or near the level of detection. One of these animals had a low viral set point at initiation of triple therapy, such that the measurable decline in viremia on ART was only a 1.2 $\log_{10}$ reduction, before plasma virus levels became undetectable. The other animal showed a greater than 4 $\log_{10}$ decrease in viral load. The third animal in this group had the highest viral load prior to ART, which declined continuously over the 20-week period by more than 4 $\log_{10}$ but which remained at detectable levels.

The five animals on ART that did not fail therapy showed constant low or undetectable viremia while on treatment. To assess the persistence of RT-SHIV$_{mne}$, a 3- to 5-week interruption in therapy was performed. Rapid viral rebound was detected after cessation of therapy for all five animals, indicating that virus-producing cells persisted during the 20 weeks of therapy. Daily ART was restarted when plasma viral loads rose to ≥3,000 vRNA copies/ml and was continued for 3 to 5 additional weeks, during which plasma virus again declined, indicating the effective absence of multidrug-resistant virus from the persisting population.

To understand the effectiveness of therapy in suppressing immunodepletion in RT-SHIV-infected animals, $CD4^+$ T-cell counts were monitored closely in the ART-treated animals prior to, during, and after triple therapy (Fig. 2C). Prior to infection, all animals had $CD4^+$ counts of 800 to 1,500 cells/μl. In the first 13 weeks of infection prior to any drug treatment, 4/6 of the animals had noticeable $CD4^+$ declines to below 250 cells/μl. The two animals without decreasing $CD4^+$ counts (M04007 and M03430) also had low plasma viremia, again confirming the correlation between RT-SHIV$_{mne}$ replication and $CD4^+$ T-cell loss. After initiation of ART, numbers of peripheral $CD4^+$ T cells increased for most animals. Interestingly, animal M03250's CD4 counts began to increase with initiation of ART but declined when the animal failed therapy and viremia rebounded.

Failure of ART in M03250 due to both NNRTI- and NRTI-associated resistance mutations. As mentioned above, animal M03250 experienced early virologic failure. Virus from this animal was studied in great detail to evaluate the evolution and kinetics of drug resistance-conferring mutations arising in RT. ASP was performed for multiple alleles from viral plasma samples taken prior to and following EFV monotherapy. This animal received EFV monotherapy, and detectable K103N amino acid substitutions were found in RT after EFV monotherapy and prior to initiation of combination ART (Table 1; Fig. 3A). Both alleles encoding K103N, AAC and AAT, were detected at similar levels 1 week after EFV monotherapy was initiated (8% and 13%, respectively) and were sustained at those levels until triple therapy was initiated. The frequencies

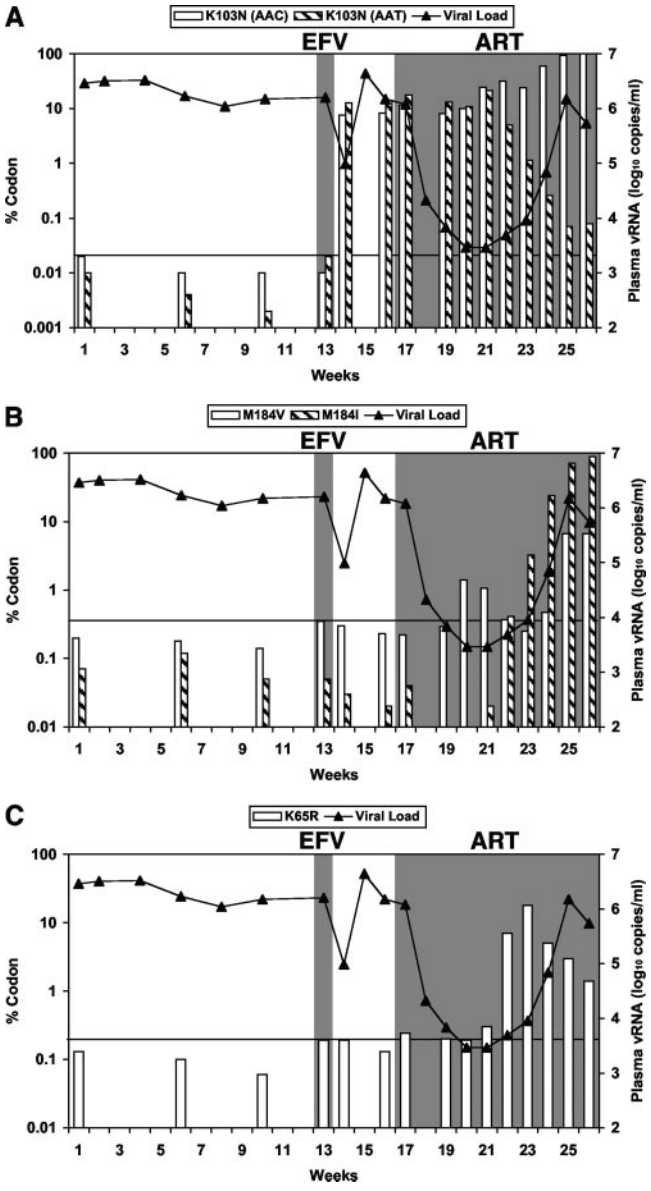

FIG. 3. Frequency of drug resistance-conferring mutations for animal M03250 prior to and during therapy. Bars indicate frequencies of K103N (AAC, open; AAT, striped) (A), M184I/V (M184I, striped; M184V, open) (B), and K65R (C), as determined by ASP. The plasma viral load is shown as solid triangles. Gray shading represents therapy administration. The thin line represents the background for each ASP assay.

of the two alleles at residue 103 did not change dramatically until 4 weeks into ART, when they doubled. At 5 weeks post-therapy (22 weeks postinfection), the amount of AAC began to rise and the AAT level declined (Fig. 3A). This trend was correlated with a rise in viral load containing the resistance-conferring mutations, confirmed by SGS (Table 2).

ASP was also performed to determine the frequencies of mutations that confer resistance to the other components of the therapy, including M184I/V, as well as K65R in virus from M03250. Six weeks after triple therapy began, M184I was detected in 3% of the plasma virus (Fig. 3B), increasing in sub-

TABLE 2. Frequencies of resistance-conferring mutations in RT of virus from macaque M03250 before and during treatment as determined by SGS[a]

Wk postinfection	Wk post-initiation of treatment		Frequency of mutation (%)[b]					
	EFV	ART	K65R[c]	L100I	K103N AAC	K103N AAT	M184I[c]	M184V[c]
13	0	0	—	—		—	—	—
17	4	0	—	2	10	5	—	—
19	NA[d]	2	—	—	17	—	—	—
22	NA	5	3	—	19	5	—	—
23	NA	6	24	—	42	3	9	—
24	NA	7	—	—	43	—	42	—
25	NA	8	2	—	95	—	89	—
26	NA	9	—	—	98	—	93	5

[a] Approximately 35 separate genomes were analyzed for each time point.
[b] —, none detected.
[c] Drug resistance-conferring mutation genetically linked to K103N (AAC) on all genomes.
[d] NA, not applicable.

sequent weeks to 88%. M184V was detected at a level of 1% at 3 weeks posttherapy, which declined to background levels, reemerging to 7% at 9 weeks posttherapy. Both M184I and M184V confer resistance to FTC (17, 19). In addition, K65R, which is associated with TDF resistance (56), was detected at some time points during failure and declined over time (Fig. 3C; Table 2). Interestingly, the amino acid substitutions M184I, M184V, and K65R were encoded only on the genomes containing the AAC allele but neither the AAT nor the wild-type AAA allele of K103.

To understand the kinetics of evolution of the virus in vivo, diversity (as average pairwise distance) of the RT coding region from single viral genomes sequenced from animal M03250 was measured before, during, and after treatment (Fig. 4). RT from the virus challenge stock had a diversity of 0.1%, which was essentially unchanged in the plasma virus after 1 week postinfection. By 13 weeks, prior to EFV monotherapy, the diversity of RT in this animal had risen to 0.43% and was maintained at this level through 19 weeks. As the viral load began to increase at weeks 22 and 23, the diversity rose to 0.52% and 0.64%, respectively. This increase was not observed if sites of resistance-conferring mutations were excluded from the calculation (Fig. 4), indicating the contribution to this effect. As the viremia continued to rise and resistance-conferring mutations at positions 103 and 184 increased at weeks 24 to 26 (Fig. 3A and B), diversity declined sharply, consistent with overgrowth by the relatively clonal mutant virus.

K103N frequency in an animal that did not fail ART: M04008. Animals M04008 and M03250 had similar levels of K103N AAC and AAT substitutions in their plasma virus. However, M04008 did not fail ART after 20 weeks. Multiple plasma samples were evaluated during therapy for the frequency of K103N in this animal by ASP (Fig. 5). Two weeks after EFV monotherapy was administered, this animal had 18% AAC and 21% AAT K103N codons. These frequencies decreased slightly at the initiation of ART, to 8% and 16%, respectively. During the 20 weeks of ART, the amount of K103N rose to 35% (AAC and AAT). After the treatment interruption and an additional 5 weeks of ART, the frequency

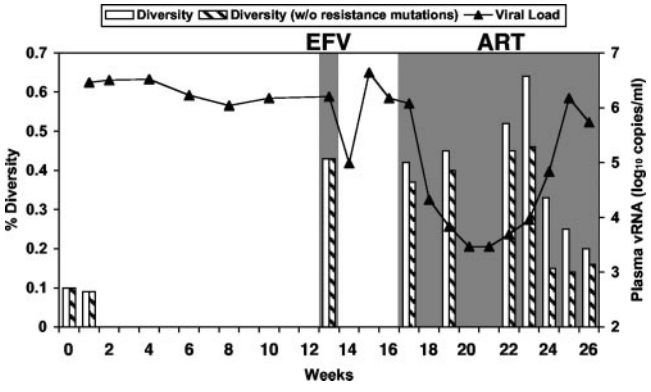

FIG. 4. Diversity of the RT coding region of viral genomes at multiple time points from animal M03250. The average pairwise difference per site for 35 genomes at each time point is represented by open bars, and diversity with drug resistance-conferring mutations removed from analysis is represented by striped bars. Plasma viral load is represented by solid triangles. Gray shading represents therapy administration.

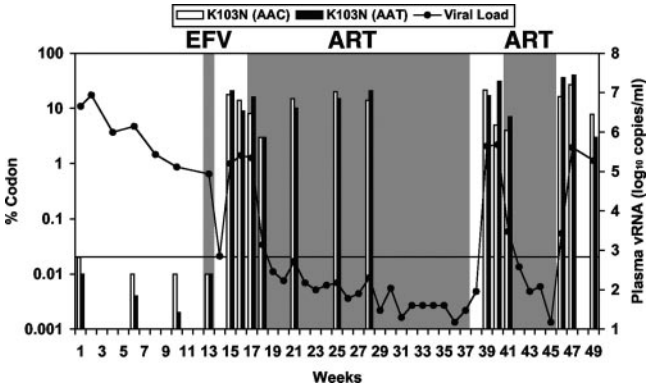

FIG. 5. Frequency of drug resistance-conferring mutations for animal M04008 prior to, during, and after therapy, as determined by ASP. The frequencies of K103N AAC (open) or AAT (solid) are plotted as bars. Solid circles indicate the plasma viral load. Gray shading represents therapy administration. K103N AAC and AAT were not detected in plasma prior to week 13. The background level for the ASP K103N assay is 0.02%. Gray shading represents therapy administration.

of K103N again increased, to 67% in this animal. No detectable M184I/V or K65R amino acid substitutions were detected in the plasma virus at the same time points (data not shown).

None of the other ART group animals had detectable drug resistance-conferring mutations, as measured by ASP and SGS at 1, 13, and 39/40 weeks (Table 1).

DISCUSSION

Although experimental infection of macaques with SIV has provided key insights into HIV-1 infection and pathogenesis in humans, few studies have used a pathogenic virus that also is sensitive to commonly used antiviral agents to investigate questions relating to therapy, persistence, and the evolution of viral resistance. Whereas studies evaluating therapy in HIV-1-infected individuals have the advantage of patient numbers, the evolution of virus during therapy can be evaluated in extensive detail within an animal model, even if the number of subjects is necessarily limited. Thus, short-lived evolutionary changes that precede more generalized selection phenomena may be better detected within an animal model. Moreover, one can observe the performance of a well-defined, consistent virus stock under different selection circumstances in different animals. Most obviously, antiviral treatment using animal models can also be manipulated in ways not ethically permissible with patients.

Despite the inherent advantages in using animal models to complement and extend studies performed with HIV-infected patients relating to therapy, studies using SIV cannot take advantage of antiviral inhibitors that specifically target HIV-1 proteins, and SIV chimeric viruses that encode large regions of HIV-1 are often limited in pathogenicity (1, 49). Experiments with RT-SHIV$_{mac239}$, a direct antecedent to RT-SHIV$_{mne}$, first demonstrated that substitution of SIV RT with HIV-1 RT yielded a virus with the desirable characteristics of robust replication within animals and susceptibility to antiviral agents which structurally recognize an HIV-1 protein (44, 51). We developed a similar infection model with pigtail macaques to investigate questions of viral dynamics and the evolution of drug resistance in response to therapy. Unlike RT-SHIV$_{mac239}$, RT-SHIV$_{mne}$ was derived from a virus with a genome that encodes a canonical tRNA^{Lys3} primer binding site and with a cellular tropism that includes macrophages in addition to CD4$^+$ T lymphocytes (2, 32, 55).

In this study, we observed that RT-SHIV$_{mne}$ replicates to high levels in vivo and is pathogenic in the absence of therapy. All macaques that were challenged with RT-SHIV$_{mne}$ became infected, and 3/4 of the animals in the untreated cohort showed progressive declines in peripheral CD4$^+$ T cells along with gastrointestinal and/or respiratory pathologies. We thus investigated the effect of therapy soon after acute infection by RT-SHIV$_{mne}$ within this model.

A question of particular interest was the efficiency and consequence of selecting NNRTI-associated resistance mutations by short courses of NVP monotherapy, as is widely used to prevent mother-to-child transmission. After determining that orally administered NVP did not give useful systemic drug levels in pigtail macaques, we initiated a study in which three RT-SHIV$_{mne}$-infected animals received a short course of EFV monotherapy to ascertain the effects of transient NNRTI therapy. Peak EFV levels detected in the blood of the macaques were approximately 80% of what is observed in patients receiving EFV therapy (C. Peloquin, personal communication). Three other infected animals were not treated with EFV. Similar to results seen in clinical trials of single-dose NVP (11, 14, 16, 30, 35), analysis of plasma virus after EFV treatment showed significant levels of K103N resistance-conferring mutations with both AAC and AAT alleles for two of the three EFV-treated macaques. These amino acid substitutions were not detected in samples from the untreated animals. While the sensitivity of our ASP assays did not permit determination of the preexisting 103N frequency prior to EFV exposure, the two animals for which K103N was selected with treatment had the highest levels of plasma viral load prior to treatment. The reason virus from the third animal in this group did not have any detectable NNRTI-associated resistance mutations was most likely due to low plasma viremia prior to transient EFV therapy. This reduced viral burden would presumably limit the absolute number of viruses with preexisting drug resistance-conferring mutations compared to the case with the other animals.

We then asked whether prior NNRTI experience would affect treatment outcome when all of the animals were dosed with combination therapy that included the NNRTI. While an increased frequency of antiviral failure in this scenario has been inferred from epidemiological studies (37), this prediction has not been experimentally tested where the evolution and dynamics of resistance from a defined inoculum could be carefully monitored over short intervals. We used the commonly prescribed regimen of FTC, TDF, and EFV, which is now available in a single, once-daily pill (22). One of the two EFV-pretreated animals with preexisting K103N in the plasma virus, M03250, failed therapy with increasing viremia despite continued treatment. This failure of ART within weeks of initiation allowed us to investigate in detail the genotypic changes that manifested in the plasma RT-SHIV$_{mne}$ and gave rise to resistance.

Virus from the animal that failed therapy had a mixture of AAC and AAT K103N amino acid substitutions after EFV monotherapy, but as virologic failure occurred in this animal the AAC allele became dominant. This phenomenon is characteristic of some HIV-1 individuals on EFV-containing ART (46). Notably, treatment failure was temporally correlated with increasing frequencies of K103N AAC linked to M184I amino acid substitutions in RT. This result implies that a clonal, multidrug-resistant virus population, in which M184I arose on a genome containing the K103N AAC allele, contributed to ART failure. The K65R amino acid substitution was present and linked to K103N AAC, but its frequency decreased over time, being replaced by the K103N (AAC)/M184I double mutant. Interestingly, a genome containing resistance-conferring mutations for all three agents was not observed. Instead, the amino acid substitutions at 103 and 184 were sufficient for a return of viremia to pretherapy levels before this animal was euthanized. The clonality of the resistant virus is also indicated by the finding that the genetic diversity in the RT coding region was inversely related to the amount of K103N/M184I/V virus. The detection of an apparent resistant, clonal population of plasma virus has been documented for patients on suppressive ART (5, 12, 21, 58). This observation suggests that ART selects

a limited number of viral clones containing mutations conferring resistance to at least two of the antiviral agents used.

These data do not imply that a K65R/K103N/M184I RT-SHIV$_{mne}$ genome had a selective disadvantage in animal M03250. Rather, there are a number of possibilities to explain the prevalence of the double resistance mutant. First, it is possible that viruses encoding K65R and M184I/V resistance-conferring mutations were replicating in separate locations, impairing their recombination. It may be that the K103N/M184I double mutant arose in a target cell-rich environment, facilitating its rapid expansion over genomes encoding K65R. Although K65R in the circulating vRNA decreased from a peak of 18% to 3% by week 25, the absolute number of plasma viral genomes encoding K65R increased from approximately 1.6×10^3 to 4.5×10^4 copies/ml during the same period. Despite the increased presence of K65R, it was rapidly outpaced by genomes encoding M184I by week 25 (1.1×10^6 copies/ml). This result implies, at least for this one animal, that the M184I amino acid substitution took longer to arise but gave more fitness than the K65R resistance-conferring mutation in the context of the triple therapy used. RT-SHIV$_{mne}$ is clearly susceptible to TDF in vitro (2), and the selection of K65R in animal M03250 and the continued suppression of EFV-resistant RT-SHIV$_{mne}$ within M04008 by ART indicate in vivo function. Finally, it should be noted that genotypic analysis was confined to approximately the first 600 nucleotides of the RT region, so it is possible that other amino acid substitutions present in the K103N/M184I double mutant virus enhanced fitness within its host.

The other five animals on ART did not experience virologic failure during the 20-week treatment period. The declines in plasma viremia appeared largely stabilized in these animals after this treatment period, which prompted us to investigate whether RT-SHIV$_{mne}$ would be controlled by adaptive immune responses within this model if therapy was terminated. However, after cessation of ART, plasma virus rebounded to pretherapy levels in all animals within 2 to 3 weeks, as is observed in HIV-infected people when effective therapy is removed (8, 20, 26, 43). When ART was reinitiated in the animals, viremia was suppressed to low or undetectable levels again. This result suggests that an unidentified reservoir of replication-competent, persisting virus is present in all animals. Susceptibility to suppression of replication after treatment interruption also means that the persisting viremia was largely wild type or, in the case of animal M04008, resistant to only a single drug in the combination treatment regimen. These data suggest that, as is shown with humans (25), RT-SHIV$_{mne}$ viral species generated prior to treatment can be archived, potentially in latent form. Because ART interferes with RT-SHIV$_{mne}$ pathogenesis with this model, virus drug resistance, latency, and persistence can conceivably be studied where therapy is administered for time periods extending to years, better mimicking conditions in HIV-infected persons receiving ART.

In contrast to infected cells that persist during ART, activated CD4$^+$ T cells are most rapidly depleted by HIV-1 infection, and thus the half-life of these cells can be inferred from the first-phase viremia decay after initial therapy (50). First-phase decay of RT-SHIV$_{mne}$ during ART ($t_{1/2}$ = 1.0 day) closely mimicked initial decay in HIV-1-infected persons receiving a 3-drug EFV protocol in which the $t_{1/2}$ value was 1.03 days (33). When ART was reinitiated in 5/6 animals after treatment interruption, a $t_{1/2}$ value of approximately 1.3 days for RT-SHIV$_{mne}$ was calculated based on the plasma viremia reduction observed 1 week later (not shown). As was observed for EFV-containing regimens in the ACTG A5166 study, no pharmacological lag in efficacy was seen after ART initiation in the RT-SHIV$_{mne}$-infected macaques. Notably, EFV monotherapy with our model also did not lag in suppressing viremia and was highly effective during first-phase decay.

Unlike transient EFV monotherapy, interruptions in ART did not detectably select for individual NRTI or NNRTI resistance mutations in 4/5 animals. In animal M04008, however, there appeared to be progressive selection of both K103N amino acid substitutions subsequent to therapy discontinuation, reaching a level of nearly 70% after the second interruption, consistent with continued selection pressure by persistent EFV. Frequent ART cessation and reinitiation are associated with the development of antiviral resistance in HIV-1-infected individuals (3, 6, 7, 39). A possible explanation for this apparent discrepancy is that the ART cessation periods in our study did not allow sufficient replication cycles to generate single drug resistance forms before the reinitiation of therapy but did support the increase in frequency of resistance-conferring mutations already present at detectable levels. It is also conceivable that longer ART continuation after treatment interruption would permit the emergence of a multidrug-resistant virus in this animal. Whether selection of drug resistance mutations occurs actively during suboptimally suppressive ART or the emergence of resistance-conferring mutations during ART reflects selection of preexisting resistant forms of virus is a question of significant clinical consequence that this animal model should help to answer. Sampling of different cells and tissues to measure RT-SHIV$_{mne}$ diversity and variability in macaques before, during, and after therapy may help clarify the nature and the dynamic properties of persistent viral reservoirs in ways not feasible in a clinical setting. The cellular reservoirs that give rise to antiviral resistance are likely the same that reseed infection after ART cessation.

While infection of rhesus macaques with the similar RT-SHIV$_{mac239}$ has also been evaluated for sensitivity to various ART regimens, these studies were not designed to test conditions that elicit the development of NNRTI and multidrug resistance and did not investigate virus dynamics (44, 51, 59, 61). In comparison to these studies, we used sensitive assays to detect viral loads as low as 15 copies of vRNA/ml of plasma during ART and to monitor the development of resistance-conferring mutations at $\leq$0.1% frequency. In 2/3 of the EFV-pretreated animals, NNRTI resistance rapidly emerged, suggesting that RT-SHIV$_{mne}$ had undergone sufficient rounds of replication in the pigtail macaques by 13 weeks postinfection to create a diverse viral quasispecies capable of resisting and adapting to selective pressure. Given that the half-life of decay of viremia on suppressive therapy approximates the replication cycle time of the virus (50), we estimate that about 90 replication cycles occurred between infection and the short-course EFV treatment.

In summary, we have demonstrated that infection of macaques with RT-SHIV$_{mne}$ is pathogenic and responsive to suppressive ART. Antiviral resistance mechanisms in this model mimic those found in HIV-1-infected persons receiving ART

targeting RT. Through frequent monitoring and sensitive genotypic assays, we observed the evolution and rapid emergence to dominance of a multidrug-resistant RT-SHIV$_{mne}$ genome under suppressive therapy. Animals in which infection was controlled continued to harbor drug-sensitive virus, which rapidly ascended after the cessation of therapy. These data suggest that this model will be useful to explore the emergence and evolutionary mechanisms that underlie HIV-1 drug resistance in vivo. RT-SHIV$_{mne}$ infection of macaques can be used to identify persisting reservoirs of virus during suppressive ART, which is not possible with the standard SIV model. Understanding the characteristics of these reservoirs under specific conditions of therapy, particularly the differences that exist in viruses present in the tissues as opposed to those in the blood, should help elucidate better HIV-1 treatment and eradication strategies.

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