Structured Treatment Interruptions with Tenofovir Monotherapy for Simian Immunodeficiency Virus-Infected Newborn Macaques

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We demonstrated previously that prolonged tenofovir treatment of infant macaques, starting early during infection with virulent simian immunodeficiency virus (SIVmac251), can lead to persistently low or undetectable viremia even after the emergence of mutants with reduced in vitro susceptibility to tenofovir as a result of a K65R mutation in reverse transcriptase; this control of viremia was demonstrated to be mediated by the generation of effective antiviral immune responses. To determine whether structured treatment interruptions (STI) can induce similar immunologic control of viremia, eight newborn macaques were infected with highly virulent SIVmac251 and started on a tenofovir STI regimen 5 days later. Treatment was withdrawn permanently at 33 weeks of age. All animals receiving STI fared much better than 22 untreated SIVmac251-infected infant macaques. However, there was a high variability among animals in the viral RNA set point after complete drug withdrawal, and none of the animals was able to achieve long-term immunologic suppression of viremia to persistently low levels. Early immunologic and viral markers in blood (including the detection of the K65R mutation) were not predictive of the viral RNA set point after drug withdrawal. These results, which reflect the complex interactions between drug resistance mutations, viral virulence, and drug- and immune-mediated inhibition of virus replication, highlight the difficulties associated with trying to develop STI regimens with predictable efficacy for clinical practice.

Although highly active antiretroviral therapy (HAART) has led to significant advances in the treatment of human immunodeficiency virus (HIV) infection, it is currently not able to eradicate infection, and the prolonged use of these drugs is often associated with problems of costs, compliance, toxicity, and resistance (52). Withdrawing drug treatment usually leads to rapid viral rebound and eventual disease progression (8). Therefore, several years ago, the concept of structured treatment interruption (STI) was proposed as an alternative to continuous HAART.

As reviewed elsewhere (1, 33, 40), STI can be used for different goals. STI may be useful (i) to limit problems of cost, chronic toxicity, and adherence by use of short-cycle intermittent treatment and by maintaining drug-mediated control of virus replication (with drug-free periods too short to allow viral rebound) (16, 18) or (ii) to replace resistant virus with wild-type virus in patients with detectable viremia before starting salvage therapies (10, 21). The third rationale for STI, which has received most attention, is based on the hypothesis that viral rebounds during serial periods of drug withdrawal may gradually enhance antiviral immune responses and reduce subsequent viral rebounds; through better immunological control of virus replication, antiviral drug treatment can be given intermittently and eventually be withdrawn for prolonged periods. However, clinical trials found that the likelihood of STI to induce immunologic control of HIV replication was generally most favorable for patients who were started on HAART during acute infection, when the immune system was still relatively intact and when early treatment was able to lower the viral RNA set point after drug withdrawal (30, 50). When treatment was started during chronic HIV infection, STI regimens were generally able to augment anti-HIV CD8+ cell-mediated immune responses (to approximately pretreatment levels) but were less effective in restoring and maintaining HIV-specific CD4+ cellular immune responses, and treatment interruptions generally led to a viral rebound to near pretreatment levels (17, 20, 44, 45, 47–49, 51).

Because HIV disease progression is usually faster in infants than in adults, it has not been demonstrated conclusively whether treatment interruption strategies can also work in a pediatric population (39). Simian immunodeficiency virus (SIV) infection of newborn rhesus macaques is a useful animal model of pediatric HIV infection and AIDS for exploration of such STI intervention strategies. We have previously shown that short-term tenofovir treatment (14 to 60 days) started 5 days after oral SIVmac251 inoculation was able to reduce viremia and enhance antiviral immune responses, but once drug treatment was stopped, virus levels still increased gradually to levels that eventually still led to disease progression (62). We have also demonstrated that prolonged treatment of SIVmac251-infected infant macaques with tenofovir leads to the emergence of SIV mutants with reduced in vitro susceptibility to tenofovir, associated with a lysine-to-arginine substi-
tution at codon 65 (K65R) of reverse transcriptase (RT) (60). The emergence of such K65R viral mutants did not always lead to an increase in viremia, as some animals were able to suppress K65R viremia to low or undetectable levels for many years (i.e., approximately 4 to 10 years) due to the development of strong CD8+ cell-mediated immune responses (60, 61, 72). However, abrupt cessation of tenofovir treatment for these animals still resulted in an increase in viremia (72).

In the present study, we investigated whether an STI regimen with tenofovir, started early during infection, would be able to induce long-term immunologic control of virus replication in this newborn macaque model. Although none of the animals was able to achieve long-term immunologic control of viremia in the absence of tenofovir treatment, this report provides further insights into the complex interaction of factors and events that occur during STI.

MATERIALS AND METHODS

Animals and sample collection for monitoring infection. All rhesus macaques (Macaca mulatta) were from the type D retrovirus-free and SIV-free colony at the California National Primate Research Center. The newborn and infant macaques were hand reared in a primate nursery. Animals were housed in accordance with American Association for Accreditation of Laboratory Animal Care standards. We adhered to standards outlined in the “Guide for Care and Use of Laboratory Animals” (43). For blood collections, animals were immobilized with ketamine-HCL (Parke-Davis, Morris Plains, NJ) (10 mg/kg of body weight) administered intramuscularly. EDTA-anticoagulated blood samples were collected regularly for monitoring immunologic and viral parameters and complete blood cell counts according to methods previously described (63, 68). Genetic assessment of the major histocompatibility complex class I alleles Mamu-A*01 and Mamu-B*01 was performed using a PCR-based technique (19, 28). In the present study, because animals were randomly assigned at birth, the frequency of the Mamu-A*01 and Mamu-B*01 alleles was similar to that among our rhesus macaque colony (∼25%). Similarly to observations in previous studies with our SIVmac251 stocks (22, 58, 72), there was no association in the present study between the presence of the major histocompatibility complex class I alleles Mamu-A*01 and Mamu-B*01 and the plasma RNA levels and disease progression (data not shown).

Experimental animal groups and virus inoculation. Within 3 days after birth, 11 newborn macaques were inoculated orally with two doses of virulent uncloned SIVmac251 (on two consecutive days) under conditions of ketamine anesthesia, according to methods described previously (62). Each dose consisted of 1 ml of undiluted SIVmac251 (of a stock designated by lot number 5/98), containing 105 SIV RNA copies per ml (63). Within 3 days after birth, 8 animals received the SIVmac251 inoculation (as described below), while three animals were untreated control animals. Those three animals had high viremia and a rapid disease course (AIDS at 11, 14, and 15 weeks of age) indistinguishable from that of 19 historical untreated newborn animals infected orally with a previous lot number of this SIVmac251 stock (8/95) and described previously (59, 62, 65, 68, 71). Accordingly, the data from studies of these 22 untreated control animals were pooled for the analysis of comparison with the drug-treated animals.

Preparation and administration of tenofovir. Tenofovir (Gilead Sciences) was suspended in distilled water, dissolved by the addition of NaOH to a final pH of 7.0 at 60 mg/ml, sterilized using a 0.2 μm filter (Nalgene), and stored at 4°C. Tenofovir was administered subcutaneously into the back of the animal. The dosage was adjusted weekly based on weight. At 5 days after the first SIVmac251 inoculation, the eight newborn macaques were started on tenofovir treatment (30 mg/kg/day administered subcutaneously once daily). After 6 weeks of tenofovir treatment, treatment was interrupted for 1 week. Tenofovir treatment was then given intermittently, each time for 4 weeks, with treatment interruptions of increasing length (2, 3, and 4 weeks) followed by complete withdrawal of drug treatment at 33 weeks of age.

Virus isolation and quantitation of plasma viral RNA. Infectious virus was isolated in cultures of peripheral blood mononuclear cells (PBMC) with CEMx174 cells and subsequent p27 core antigen measurement via an enzyme-linked immunosorbent assay (ELISA), according to methods previously described (66). Viral RNA in plasma was quantified using version 3.0 of a bDNA signal amplification assay specific for SIV that has a lower quantitation limit of 500 copies per ml of plasma (68).

Phenotypic drug susceptibility assays. Phenotypic drug susceptibilities to tenofovir of SIV isolates were characterized by a previously described assay (based on a dose-dependent reduction of viral infectivity) that was able to detect viral mutants with reduced susceptibility to tenofovir (60, 70).

Sequence analysis of SIV RT-encoding region of virus isolates. DNA sequence analysis of the codons 60 to 320 of RT were performed on proviral DNA obtained from CEMx174 cells infected with virus isolated from the SIV-infected animals. Infected cells were harvested as soon as culture supernatant results were positive by p27 antigen capture ELISA. Genomic DNA was extracted and used for nested PCR according to methods and with primers described previously; amplicons were sequenced by Davis Sequencing (Davis, California) with primers 239-2786 and SIV-RT3 (67, 72). This method can detect the presence of a 20% subpopulation.

Real-time PCR to quantitate the frequency of K65R in SIV RNA in plasma. The generally used real-time PCR methodology has previously been described elsewhere for HIV type 1 (HIV-1) drug resistance testing (25) and for SIV (26). Briefly, the sensitive, real-time PCR assay for SIVmac K65R mutations involves the comparison of PCR amplifications between a total virus copy reaction and a 65R mutation-specific reaction, such that a sample with 100% K65R mutant virus would have similar amplification levels (Cₚ values) for the total copy and mutation-specific reactions. For real-time PCR, a 627-bp region of the viral RNA (RT nucleotides 80 to 706) was first amplified by RT-PCR. The RT reaction conditions were 39°C for 1 h and then denaturing at 95°C for 5 min. PCR amplifications were repeated for 40 cycles of melting at 94°C for 1 min, annealing at 50°C for 30 s, and extension at 72°C for 1 min.

The real-time PCRs for the total virus copy and K65R mutation were performed in duplicate using a 6-carboxyfluorescein-labeled probe with an internal biochromo-quencher that merely acts as a reporter when degraded during forward primer extension.

Evaluation of the real-time PCR primers against plasms and virus plasma sequences possessing either the wild-type 65R or the mutant 65R SIV codon revealed that a difference in total copy and mutation-specific amplifications (ΔCₚ) of nine cycles or fewer (ΔCₚ ≤ 9 cycles) signified the presence of the K65R mutation. This assay cutoff allowed mutant viruses to be detected at frequencies between 0.1 and 100%.

Detection of SIV-specific immune responses and interleukin-12 levels. The ELISA to detect SIV-specific immunoglobulin G (IgG) was performed as described previously (71). The number of antigen-specific gamma interferon (IFN-γ)-producing cells was measured using an enzyme-linked immunospot assay described previously (46) and a pool of 15-mer peptides with a 10-amino-acid overlapping of the entire p24 gag region of SIVmac239; results were considered positive when the number of spot-forming cells (SFC) for 2 × 105 cells was 10 per million greater than the mean background (background values for the SIVmac239 only) plus two standard deviations. Plasma levels of interleukin-12 (IL-12) were measured using a rhesus macaque-specific IL-12 ELISA kit (U-CyTech, Utrecht, The Netherlands) according to manufacturer's instructions.

Neutralizing antibody assay. Neutralization was measured as reductions in luciferase reporter gene expression after multiple rounds of virus replication in 5.25.EGFP-Luc.M7 cells (41). This cell line is a genetically engineered clone of CEMx174 that expresses multiple entry receptors (CD4, CXCR4, GPR15/Bob) and was transduced to express CCR5 (5). The cells also possess Tat-responsive reporter genes for luciferase and green fluorescence protein (GFP). Cells were maintained in growth medium (RPMI 1640, 12% heat-inactivated fetal bovine serum, 50 μg gentamicin/ml) containing puromycin (0.5 μg/ml), G418 (300 μg/ml), and hygromycin (200 μg/ml) to preserve the CCR5 and reporter gene plasmids. For the neutralization assay, 5 × 10⁴ SIV-infected cells were incubated with multiple dilutions of test sample in triplicate for 1 h at 37°C in a total volume of 150 μl in 96-well flat-bottom culture plates. A 100 μl suspension of cells (5 × 10⁵ cells/ml of growth medium containing 25 μg DEAE dextran/ml but lacking puromycin, G418, and hygromycin) was added to each well. One set of control wells received cells and virus (virus control), while another set received cells only (background control). Plates were incubated until approximately 10% of cells in virus control wells were positive for green fluorescence protein expression by fluorescence microscopy (approximately 3 days).

At this time, 100 μl of cell suspension was transferred to a 96-well white solid plate (Costar) for measurements of luminescence using Bright Glo substrate solution as described by the supplier (Promega). Neutralization titer values represent the dilution at which relative luminescence units were reduced by 50% compared to virus control well results after subtraction of background relative luminescence units. Cell-free stocks of tissue culture laboratory-adapted
(TCLA)-SIVmac251 and primary SIVmac251 were generated in H9 cells and CEM174 cells, respectively.

**Lymphocyte phenotyping.** Three-color flow cytometry techniques were used to measure CD3, CD4, CD8, and CD20 antigens according to methods described previously (71). Additional antibodies that were used include anti-CD16-phycoerythrin (clone 3G8; BD-Pharmingen), anti-CD8-PE (clone G46-6; BD-Pharmingen) and anti-CD5-PE (clone ALB11; Beckman Coulter). CD4+ T lymphocytes, CD8+ T lymphocytes, B lymphocytes, and NK cells were defined as CD4+CD3+, CD8+CD3+, CD20+CD3-, and CD8+CD3- lymphocyte populations, respectively.

**Criteria for euthanasia and animal necropsies.** Euthanasia of animals with simian AIDS was indicated by clinical observations of a severe life-threatening situation for the animal that have been described previously (71). A complete necropsy with a routine histopathologic examination of tissues was performed. Tissues were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 6-μm intervals, stained with hematoxylin and eosin, and examined by light microscopy.

**Statistical analysis.** Statistical analysis of disease-free survival was done using a log rank test. Statistical analysis for correlations between viral and immunologic parameters was performed with Pearson or Spearman correlation. Viral RNA levels were log transformed for all statistical analyses. All analyses were performed using Prism Version 4.0 for Mac (GraphPad Software Inc. San Diego, CA).

**RESULTS**

**Outcome of oral SIVmac251 infection in untreated newborn macaques.** Infant rhesus macaques were inoculated orally within 3 days of birth with virulent uncloned SIVmac251 (Fig. 1). Twenty-two animals were untreated control animals and had persistently high viremia and a rapid disease course (median AIDS-free survival time of 12 weeks) (Fig. 2 and 3A). Seventeen of the 22 untreated animals had a viral RNA set point of more than 7 log RNA copies per ml (with viral set point defined as the highest viral RNA level in plasma at 6 to 8 weeks after virus inoculation) (71). Similarly to findings of previous studies (60, 62, 63, 68, 70), this high viremia in untreated SIVmac251-infected infant macaques was associated with transient or poor anti-SIV antibody responses (SIV-specific IgG titers, ≤6,400; data not shown).

**Structured treatment interruptions with tenofovir during SIVmac251 infection: summary of virologic and clinical outcome.** Eight newborn rhesus macaques were inoculated orally with SIVmac251. Five days after virus inoculation, when all animals had detectable viremia (3.5 × 10^3 to 1.0 × 10^7 SIV RNA copies/ml plasma), they were started on a tenofovir STI regimen (Fig. 1). Seven weeks after virus inoculation (i.e., after ~6 weeks of tenofovir treatment), tenofovir treatment was interrupted for 1 week and then reinitiated with consecutive constant treatment periods of 4 weeks but gradually increasing drug-free periods (2, 3, and 4 weeks). Tenofovir treatment was discontinued permanently at 33 weeks. The efficacy of the STI regimen in reducing viral replication was highly variable (Fig. 3B, Fig. 4). For some animals (e.g., animal 32086; Fig. 4A), this STI strategy gave reasonable virologic results, as the viral rebound gradually decreased with consecutive treatment interruptions; for other animals (e.g., animal 32102; Fig. 4H), viremia rebounded to high levels during each treatment interruption, and eventually the tenofovir treatment courses had little impact on reducing viremia. For ease of further data analysis and discussion, the STI animals were stratified according to their viral RNA levels at 43 weeks of age (i.e., 10 weeks after permanent discontinuation of tenofovir treatment), when their viral RNA set points (the number of SIV RNA copies per milliliter of plasma) ranged from <5 log (one animal) to 5 to 6 log (two animals), 6 to 7 log (two animals), and >7 log (three animals) (Fig. 4).

Despite mixed results with respect to lowering posttreatment viral set points, tenofovir STI was still beneficial for prolonging disease-free survival in comparison to the results seen with the untreated animals (log rank test, \( P < 0.0001 \)) (Fig. 2). All STI animals were alive at 50 weeks of age, at which time the five animals with highest viremia were euthanized; the three animals with a log viral RNA set point above 7 (animals 32102, 32122, and 32125) had histopathologic evidence of the onset of opportunistic infections (such as candida and/or crypt-
tosporidium infections; data not shown). The remaining animals with a lower set point (<6 log RNA copies/ml plasma) remained healthy and asymptomatic throughout the observation period (to 65 weeks of age).

(i) Predictors of virologic set point. Because the viral RNA set point at 43 weeks of age was quite variable (from <5 to >7 log RNA copies/ml), we attempted to determine which early markers would be predictive of this virologic outcome.

(ii) Early viral RNA levels. Viral RNA levels 5 days after oral SIVmac251 inoculation (i.e., at the start of tenofovir treatment) ranged from 3.5 × 10^7 to 1.0 × 10^8 RNA copies/ml plasma. As indicated in Fig. 5, there was no correlation between viral RNA levels at the start of treatment (day 5) and the subsequent viral set point. A similar lack of correlation was observed for the time points during the first tenofovir treatment period (to 65 weeks of age). It was only later, especially from week 12 (i.e., the end of the second treatment period) onward, that the stratification of the different groups, based on the final viral RNA set point, became more evident (week 12; Pearson correlation, \( P = 0.02 \)) (Fig. 5). When instead of absolute viral RNA levels, slopes of viral RNA changes were analyzed during and between treatment periods, no obvious correlation was found with the final viral RNA set point (data not shown).

Emergence of drug resistance mutations. Previous studies have demonstrated that in tenofovir-treated SIV-infected macaques, the K65R mutation in RT, which confers approximately fivefold-reduced susceptibility to tenofovir in vitro, is the primary mutation (60, 61, 71, 72). In the present study, the emergence of K65R SIV mutants was monitored by two techniques. Virus isolated from PBMC was used for population sequence analysis of the RT region to detect K65R and other mutations in RT (codons 0 to 320). In addition, sensitive real-time PCR was used to quantitate the percentage of K65R virus in viral RNA populations in plasma samples with sufficient virus levels (≥4,000 RNA copies per ml) that were collected early during the study (i.e., until K65R was detected in virus isolates from PBMC by sequencing). Both assays demonstrated that virus of all tenofovir-treated animals developed the K65R mutation within 2 to 10 weeks of infection (i.e., approximately 1 to 9 weeks after the start of the first tenofovir treatment course) (Fig. 4). Due to different detection limits for infectious virus in PBMC and viral RNA in plasma, both techniques could not always be applied to samples of the same time point, but the detection of K65R in plasma by use of sensitive real-time PCR either preceded or coincided with the detection of K65R in virus isolated from PBMC (six and two animals, respectively) (Fig. 4). This earlier detection of drug resistance in virus in plasma relative to virus isolated from PBMC (which includes archived provirus) is consistent with observations of studies conducted with HIV-infected humans (27). Five of the eight animals had detectable K65R SIV mutants in plasma within 7 weeks of age (i.e., prior to the first treatment interruption). For these five animals, the 1-week tenofovir treatment interruption at 7 weeks resulted generally in a decrease in the percentage of K65R mutants in plasma RNA populations at week 8, indicating selective outgrowth of wild-type virus over the early K65R viral mutants in the absence of drug (for example, see the results for animals 32086 and 32078 in Fig. 6). Reinitiation of tenofovir therapy at week 8 led to a reduction of viral RNA levels, including those of K65R SIV RNA, which indicated that tenofovir therapy was also associated with antiviral effects against K65R SIV (Fig. 6).

A rapid emergence of K65R mutants was not predictive of a poor virologic response as determined by (i) viremia during periods of tenofovir treatment or (ii) viral RNA set point after permanent cessation of treatment (Fig. 4 and 6). Instead, although group sizes based on viral RNA set point were small, we observed a trend for a slower detection of K65R SIV mutants in plasma viral RNA and virus isolates from animals with poorer outcome (i.e., higher viral RNA set points) (Fig. 7). For example, for animal 32086 (an example of a good virologic responder), K65R SIV mutants already became detectable (10% K65R) in plasma viral populations after 2 weeks of infection (i.e., only 9 days of tenofovir treatment); but despite this presence of K65R virus, viremia was reduced each time tenofovir treatment was reinitiated, the viral rebounds during treatment interruption became gradually smaller, and the final viral RNA set point was low (<5 log) (Fig. 4A and 6). In contrast, for animal 32102 (an example of a poor responder),

FIG. 3. Virus levels in untreated and tenofovir-treated animals. Virus levels in 22 untreated newborn animals (A) and 8 tenofovir STI animals (B) were measured by SIV bDNA assay. In panel B, animals are stratified based on their viral RNA set points (i.e., SIV RNA levels per milliliter of plasma at 43 weeks). Horizontal lines are given for ease of comparison.
FIG. 4. Virus levels, genotypic drug resistance, and CD4⁺ T-lymphocyte data. Panels A through H show the individual plasma viral RNA levels (left y axis) and percentages of CD4⁺ CD3⁺ T lymphocytes in peripheral blood (right y axis) for the eight tenofovir STI animals (arranged according to their viral RNA set points, i.e., SIV RNA levels per milliliter of plasma at 43 weeks) (see Fig. 3B). RT sequencing results for virus isolated from PBMC are indicated at the tested time points in the rectangular boxes; wt indicates the wild-type sequence. The percentage of the K65R mutation in plasma viral RNA populations, measured by real-time PCR, is indicated below the x axis for the early time points.
the K65R mutation was detected in plasma viral RNA relatively late (10 weeks of infection) but the STI regimen was less effective, because once K65R mutants were detected, reinitiation of tenofovir therapy had little or no effect on reducing viremia and the final viral RNA set point was high (\(7 \log\)).

For most other animals (e.g., animals 32078 and 32125), the virologic implications associated with the detection of K65R SIV mutants changed over time, as early during infection, the reinitiation of tenofovir therapy was associated with a reduction in viremia, while later in infection, the virologic response to tenofovir therapy faded.

Similarly to observations in our previous studies (60, 61, 69, 71, 72), the detection of K65R in virus isolates coincided with or was followed by the development of other mutations in RT (such as K64R, N69S, I118V, and S211N), which are thought to be compensatory mutations (as they do not further decrease the in vitro phenotypic susceptibility). The development of these additional RT mutations (the time of first detection, specific mutations, or number of mutations) did not have any obvious correlation with the viral RNA set point of the STI animals (Fig. 4).

(iii) Immunological parameters. In contrast to the untreated control animals, which showed weak (titer \(6,400\)) or transient anti-SIV IgG responses (see above), all eight animals receiving tenofovir treatment showed strong anti-SIV antibody responses (ELISA titer \(102,400\) by 14 weeks of infection). This association of sustained anti-SIV antibody responses with prolonged survival is consistent with previous observations in SIVmac251-infected infant macaques (58, 60, 62, 63). The time needed to reach a titer of 12,500 was used as a measure of the kinetics of the anti-SIV IgG response in the tenofovir-treated animals. A faster SIV-specific IgG response correlated with higher viral RNA levels at day 5 (i.e., more antigenic stimulation at the onset of tenofovir treatment; Spearman rank correlation, \(P = 0.02\)) (Fig. 8A). The rate of the antiviral antibody response did not correlate with the final viral RNA set point of the drug-treated animals (Fig. 8B).

Despite these high titers of SIV-binding antibodies in the tenofovir-treated animals, these antibodies had little or no neutralizing activity (Table 1). Low neutralizing activity against laboratory-adapted SIVmac251 was detected only in plasma of the two animals that had a viral setpoint of 5 to 6 log (i.e., the two animals that also showed the earliest SIV-binding IgG response) (Fig. 8B); neutralizing antibody titers against primary SIVmac251 did not correlate with the viral RNA set point (Table 1).
To measure SIV-specific cell-mediated responses, an IFN-γ enzyme-linked immunospot assay with SIVgag peptides was performed on cryopreserved PBMC samples. No responses above the cutoff value (50 SFC per million cells) were detected in PBMC during the first 3 months of life. This difficulty of detecting SIV-specific IFN-γ-producing cells in PBMC is consistent with our previous observations of SIVmac251-infected infant macaques (58, 63). Quantitation of SIV-specific IFN-γ-producing cells in PBMC collected at 1 year of age was also not informative, as almost all animals (including animal 32086, which had the lowest viral RNA set point) had undetectable levels; an exception was animal 32065 (295 SFC per million PBMC), which had a set point of 5 to 6 log viral RNA copies/ml.

Flow cytometry analysis of lymphocyte surface markers was performed on whole blood. Similarly to findings in previous studies, absolute counts of lymphocyte subsets had high variability (due to variable total lymphocyte counts), so the percentages were a more reliable marker (63, 71). Of all the cell types measured in the present study, the CD4+ T lymphocyte percentage was found to correlate the best with the viral RNA set point and clinical outcome. Previously published studies have demonstrated a natural age-related decrease in the percentage of CD4+ T lymphocytes in uninfected infant macaques that is further accelerated by SIVmac251 infection (13, 71). In the present study, the CD4+ T-lymphocyte percentage was most stable for animals with a lower viral RNA set point (<6 log) (animals 32086, 32065, and 32240), with values generally remaining above 30% for the first year of life, while animals with a high viral RNA set point (>6 log) demonstrated an earlier reduction in the percentage of CD4+ T lymphocytes (<30%) (Fig. 4). For some animals (e.g., animals 32086, 32065, 32240, and 32124), CD4+ T-lymphocyte percentages fluctuated, often showing transient increases early after reinitiation of tenofovir treatment (Fig. 4).

Because tenofovir primed rhesus PBMC in vitro for enhanced IL-12 secretion following exposure to bacterial antigen (64), we measured IL-12 levels in plasma samples of the current STI study. While all plasma samples had detectable IL-12 (range, 177 to 2,550 pg/ml), there was large temporal and individual variability and no correlation was found between early IL-12 levels (absolute levels and/or area-under-the-curve analysis levels) and subsequent viral RNA set points (data not shown).

FIG. 6. Early detection and kinetics of K65R SIV in plasma of tenofovir-treated infant macaques. While a summary of results for all eight treated animals is provided in Fig. 4, data for four animals for which most data were available are presented here in more detail. Total SIV RNA levels in plasma were determined by SIV bDNA assay. For samples with sufficient total SIV RNA (>4,000 copies/ml; horizontal dashed line), the percentage of K65R virus was measured by real-time PCR and is indicated. K65R virus levels were calculated from the percentage of K65R virus multiplied by the total SIV RNA levels (K65R results above 80% were given a value of 80% for this calculation). Shading indicates periods of tenofovir treatment.
DISCUSSION

The present study is, to our knowledge, the first to test an STI regimen in a newborn macaque model. The advantage of an animal model is that it allows control of certain variables. The results of our study demonstrate that despite close control of several input parameters (such as the age of the animals, the virus inoculum, and the schedule of the STI regimen), the outcome was quite variable, due to a complex interaction of host and viral factors that were difficult to anticipate or predict early during infection.

In the present study, SIVmac251-infected infant macaques were started on a tenofovir STI regimen 5 days after virus inoculation. The treatment was beneficial, as the STI animals had a better clinical and virologic outcome than untreated animals and also fared better (based on viral RNA levels) than previously described animals that had received a single treatment course with tenofovir for 14 to 60 days (62). However, even though drug treatment was started very early in infection, the outcome had considerable individual variability, and none of the animals was able to achieve long-term immunologic control of viremia to low levels in the absence of tenofovir. In contrast, we demonstrated previously that continuous tenofovir monotherapy of SIVmac251-infected macaques starting during acute viremia led to persistently low or undetectable viremia in the majority of animals, even after the emergence of K65R mutants, due to the development of strong antiviral immune responses (60, 72).

Several other macaque studies that used a single treatment course or a regimen with multiple interruptions were more effective in inducing a lower viremia set point in a larger proportion of the animals (23, 24, 29, 31, 32, 34, 54–56, 74). A combination of factors is likely to be responsible for this difference in success rate, as these other studies used (i) older animals, (ii) less-virulent virus isolates (i.e., isolates that induce lower viral RNA set points in untreated animals or that, following suppression of acute viremia, are easier to control immunologically), (iii) combination drug regimens with a longer initial treatment period, or (iv) a combination of antiviral drug treatment with immunotherapeutic strategies (e.g., immunization and adoptive cell transfer). Our study used newborn macaques, monotherapy, and highly virulent SIVmac251. Because SIVmac251 infection of newborn macaques results in persis-

FIG. 7. Lack of correlation between earliest detection of K65R mutation and viral RNA levels at the start of tenofovir treatment or at the viral set point. The y axis indicates the time (weeks of infection) when K65R was first detected in plasma viral RNA by real-time PCR (A and B) or in virus isolated from PBMC by population sequencing of the RT region (C), as presented in Fig. 4. In graphs B and C, animals are stratified on the x axis according to their final viral RNA set points (i.e., plasma viral RNA levels at 43 weeks of age). There was no correlation between log-transformed viral RNA levels at the start of tenofovir treatment (i.e., 5 days after infection) (A) or log-transformed viral RNA set points (B and C) and the time of first detection of the K65R mutation (Spearman P values, >0.3).

FIG. 8. Kinetics of anti-SIV IgG response in tenofovir-treated infant macaques. The time (weeks of virus infection) required to reach an anti-SIV IgG titer of $\geq 12,500$ is presented along the y axis as a function of the viral RNA levels in plasma at day 5 after oral SIVmac251 inoculation (Spearman rank correlation $r = -0.818; P = 0.02$) (A) and according to the animal groups stratified by viral RNA set points at 43 weeks of age (Kruskal-Wallis test; no significant differences) (B).
tently high viremia (>7 log RNA copies per ml plasma) and rapid immunodeficiency in untreated animals, this posed an extra challenge for intervention strategies aimed at inducing rapid immunologic control of virus replication. Our initial treatment course of 6 weeks was short and empirically insufficient to allow the generation and/or maturation of effective antiviral immune responses, which explains the rapid viral rebound at the first tenofovir treatment interruption. It is likely that a longer initial treatment period would have given better results.

We stratified the STI animals based on viral RNA set point after permanent discontinuation of treatment. We were not able to define any viral or immunologic parameters early in infection that were predictive of the final outcome. For HIV-infected adults receiving STI, the viral set point after treatment interruption was significantly associated with the pretreatment plasma viremia and CD4+ cell counts (20, 45, 49, 73, 77), but these people were started on treatment during chronic infection; in other words, the pretreatment virus load set point was already determined by the strength of antiviral immune responses at that time. In our infant macaque study, because treatment was started 5 days after virus inoculation, antiviral immune responses were still in very early stages, and viral RNA levels at the start of treatment or during the initial treatment course were not predictive of the subsequent virologic outcome.

In the present study, SIV-specific immune markers did not correlate with viral set point, but the study had the limitations that it did not include (i) a more detailed evaluation of cell-mediated immune responses, including those produced in lymphoid tissues, or (ii) evaluation of the emergence of CTL escape mutants (42). The difficulty of using immunologic parameters for blood to accurately predict the virologic outcome of STI is consistent with observations from human studies, where no or a variable correlation was observed between viral load markers and HIV-1-specific CD8+ or CD4+ T-cell responses that were augmented in blood during treatment interruptions (14, 17, 20, 45, 49).

The emergence of drug-resistant viral mutants has not been evaluated in the majority of STI studies in macaques. In the present study, the tenofovir STI regimen selected rapidly for K65R SIV mutants in a time frame similar to that observed for macaques with continuous tenofovir monotherapy (36, 60, 70, 71, 72). Contrary to expectations, a rapid emergence of K65R mutants was not predictive of a poor virologic response. In fact, some animals with early emergence of K65R mutants were among those that had the best virologic response as determined by (i) a reduction of viremia upon reinitiation of tenofovir therapy and (ii) the final viral RNA set point.

Findings of our previous studies with tenofovir in the macaque model suggest that a complex interaction of factors was responsible for the observations of the present STI study (57, 71, 72). These factors include (i) the effect of K65R on viral replication fitness, (ii) the role of antiviral immune responses, and (iii) a residual antiviral effect of tenofovir against K65R viral mutants.

The tenofovir-selected K65R mutation in RT reduces replication fitness of HIV-1 in vitro by ~50% (76). The K65R mutation is likely to also reduce replication fitness of SIV in vivo, because its emergence is followed by the accumulation of additional mutations in RT thought to be compensatory mutations that improve replication fitness because they do not affect the in vitro susceptibility to tenofovir (60, 71, 72). In the absence of tenofovir treatment, the in vivo replication fitness and virulence of SIV populations with K65R and other compensatory mutations were indistinguishable from those of wild-type virus (61, 69). Thus, in the present study, the STI-treated infant macaques had the challenge to cope with virus populations with various degrees of replication fitness and drug susceptibility: (i) highly virulent wild-type virus, fit to replicate well during drug-free periods, and (ii) K65R virus, which could replicate better during tenofovir treatment and became gradually more replication fit and virulent as compensatory mutations accumulated.

Another force driving viral dynamics during this STI experiment was the immune system, which has a dual role, as it provides target cells for virus replication but also tries to contain virus replication. A potential dilemma was that the STI regimen may have allowed partial immune preservation and restoration during periods of drug treatment. In the absence of sufficiently strong antiviral immune responses, the larger availability of target cells whenever treatment was interrupted may have favored increased virus replication, especially in the available SIV-specific CD4+ T cells (15), which in turn may have reduced the likelihood of achieving effective immunologic control of virus replication. This may also explain the fluctuating patterns in CD4+ T-lymphocyte percentages that were observed in some animals, similarly to observations in humans undergoing STI (49).

The role of antiviral immune responses in containing virus replication has to be considered not only during drug-free periods but also during periods of drug treatment. We have previously demonstrated an important role of antiviral immune responses, especially CD8+ cell-mediated immune responses, in reducing SIV viremia during tenofovir therapy both
at the onset of therapy (when the virus was wild type) and during chronic therapy (when virus had the K65R mutation) (72). A proposed model of viral dynamics during drug therapy, in which the half-life of productively infected cells is determined largely by the strength of antiviral immune responses, is described in more detail elsewhere (57, 72) and helps to explain several findings in the current study, including the delayed detection of K65R mutants in some animals that demonstrated poor immunologic control of viremia.

In our previous studies with macaques, we observed that although antiviral immune responses played a major role in suppressing K65R viremia, they were not sufficient, because withdrawal of tenofovir therapy resulted in a gradual increase of K65R viremia (72). In other words, tenofovir therapy was still associated with residual antiviral effects against K65R virus, and viremia could be controlled as long as there was sufficient assistance of antiviral immune responses (72).

Accordingly, the results of the current study can be explained best by noting that these different forces of (i) altered replication fitness of mutant viruses, (ii) antiviral immune responses, and (iii) residual drug activity are not mutually exclusive and stable but instead interact and are dynamic. For example, even a relatively minor decrease in replication fitness or a partial inhibition of virus replication by the drug regimen can have a major impact on viremia if it provides more opportunity for antiviral immune responses to kill productively infected cells prior to the major viral burst. In contrast, without (or during a gradual loss of) effective antiviral immune responses, such as during progressive immunodeficiency, a small difference in replication fitness or any residual drug activity would no longer translate into any significant difference in viremia.

Our results with SIVmac251-infected infant macaques are consistent with the results of studies with human children. Following HAART, younger children are less likely to have detectable anti-HIV cell-mediated immune responses in peripheral blood, have a poorer virologic response to HAART, and have more risk of developing drug resistance than older children (4, 35, 53, 75). To our knowledge, there are no published reports on successful STI regimens for HIV-infected newborns and young infants, while the results for older infants were mixed (7, 39). The Pediatric AIDS Clinical Trials Group P1015 Team studied STI in HIV-infected children of more variable outcome and the difficulty of reliably predicting the ultimate outcome of STI before interrupting treatment for individual patients. Although some patients may benefit from drug-free periods (to reduce problems of toxicity, adherence, or cost), many patients do not show the desired long-term virologic and immunologic response during STI and may benefit more from continuous treatment even after drug-resistant mutants have been detected (3, 6, 9, 11, 12, 37–39). Accordingly, future studies should continue to look for affordable, simple, and safe combination regimens that are suitable for long-term treatment of HIV-infected children or identify more predictably effective immunotherapeutic strategies that can reduce the dependence on antiviral drugs (2).

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REFERENCES

13. DeMaria, M. A., M. Casto, M. O’Connell, R. P. Johnson, and M. Rosenzweig,


