Chemoprophylaxis with Tenofovir Disoproxil Fumarate Provided Partial Protection against Infection with Simian Human Immunodeficiency Virus in Macaques Given Multiple Virus Challenges

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We examined the efficacy of tenofovir disoproxil fumarate (TDF) in blocking simian human immunodeficiency virus (SHIV) infection in Chinese rhesus macaques. Once weekly for 14 weeks or until a macaque became infected, 12 male macaques were inoculated intrarectally with amounts of SHIVSF162P3 (10 median tissue culture infective doses; virus particles) that were ∼5-fold higher than the human immunodeficiency virus type 1 RNA levels noted in human semen during an acute infection. Of the 12 macaques, 4 received oral TDF daily, 4 received oral TDF once weekly, and 4 (control animals) received no TDF. The control animals became infected after receiving a median of 1.5 virus inoculations; macaques receiving TDF daily (1 macaque remained uninfected after 14 inoculations) and those receiving TDF weekly became infected after a median duration of 6.0 and 7.0 weeks, respectively. Although infection was delayed in treated macaques, compared with control macaques, the differences were not statistically significant (P = .315); however, the study was limited by the small numbers of animals evaluated and the variability in blood levels of TDF that resulted from oral dosing. These data demonstrate that treatment with oral TDF provided partial protection against SHIV infection but ultimately did not protect all TDF-treated animals against multiple virus challenges.

There is an urgent need to expedite the assessment of new and readily available biomedical approaches to the prevention of HIV infection. Although efforts to develop vaccines and topical microbicides for the prevention of HIV infection continue, these products are years away from being proved efficacious and commercially available. One approach to the prevention of HIV infection that is being considered as a prevention strategy for persons who are at high risk for HIV infection is the use of preexposure prophylaxis (PrEP)—that is, the treatment of uninfected persons with ≥1 antiretroviral drug before HIV exposure [1–3]. One antiretroviral drug under consideration for use in PrEP is tenofovir disoproxil fumarate (TDF), the oral prodrug of tenofovir (also known as “PMPA”). Tenofovir is a competitive inhibitor of HIV-1 reverse transcriptase.

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and terminates the growing DNA chain [4]. Properties that make tenofovir an attractive drug for chemoprophylaxis are (1) its longer half-life in serum (17 h) and in cells (>60 h), compared with that noted for other nucleoside analogues; (2) its antiviral activity in diverse cell types, including resting cells; and (3) the relatively high barrier to the development of viral resistance to the drug. TDF is approved by the US Food and Drug Administration for the treatment of HIV infection in humans and is provided as a pill that is taken once daily (Viread; Gilead Sciences). TDF is well tolerated and has a low rate of associated adverse events after long-term administration [4, 5].

Several strategies have indicated that chemoprophylaxis with antiretroviral drugs may prevent HIV infection. The most compelling of these strategies has been the use of antiretroviral drugs, such as zidovudine and nevirapine, in the prevention of mother-to-child transmission of HIV [6–8]. Another successful strategy is postexposure chemoprophylaxis, which has significantly reduced the number of infections occurring among health care workers who have been exposed to HIV through contact with the blood or body fluid of HIV-infected patients [9]. Nonhuman primate studies involving postexposure prophylaxis have had mixed but generally encouraging results. In macaques, infection resulting from a single, high-dose, intravenous inoculation with simian immunodeficiency virus (SIV) was blocked when tenofovir was administered subcutaneously either 48 h before or within 24 h after exposure and then was continued daily for 28 days [10]. Treatment was not effective if initiated 48 or 72 h after exposure or if continued only for 3–10 days [11]. In a study designed to model mucosal exposure to HIV, female macaques that were given subcutaneous tenofovir treatments for 28 days, starting 12 or 36 h after vaginal exposure to HIV-2, were protected against HIV-2 infection [12]. Partial protection was observed when tenofovir treatment was initiated 72 h after exposure [12]. In another study, combined multiple-drug chemoprophylaxis (zidovudine, lamivudine, and indinavir) initiated 4 h after challenge with simian human immunodeficiency virus (SHIV) and continued for 28 days did not protect against infection [13].

Using SIV infection in newborn macaques as a model for pediatric HIV infection, a number of studies have yielded information about the efficacy of tenofovir chemoprophylaxis. Two doses (30 mg/kg) of tenofovir administered subcutaneously 4 h before and 24 h after oral inoculation with SIV protected newborn macaques against SIV infection [14]. Reducing the dose of tenofovir to 4 mg/kg but continuing administration of the drug in a 2-dose regimen resulted in protection against infection in 5 of 8 newborn macaques [15]. However, a single dose of tenofovir given to pregnant macaques just before cesarean section did not protect newborns against oral SIV challenge after birth [16]. In the same study, a regimen of postexposure treatment with tenofovir for 2 weeks, started immediately after oral inoculation of newborns with SIV, did protect the newborns against infection [16].

All the macaque studies discussed to date have used a single high-dose virus exposure. One of the first studies to examine multiple virus challenges used the infant macaque to model mother-to-child transmission of HIV through breast-feeding. Infant macaques received multiple oral inoculations with SIV and were treated daily with oral TDF, starting 1 day before the first inoculation. That one-half of the TDF-treated animals were protected against infection suggests that the use of daily TDF treatment for infants may partially protect against transmission of HIV via breast-feeding [17].

Our goal was to evaluate PrEP with TDF, by use of an intrarectal repeated-exposure model of HIV infection in adult macaques. This model attempts to approximate high-risk HIV infection in humans through multiple inoculations of macaques with levels of SHIV that are closer to the levels of HIV-1 noted in the semen of humans with acute infection, and it has been used to assess such interventions as the use of microbicides [18–22].

**ANIMALS, MATERIALS, AND METHODS**

**Animals.** We used 12 adult male Chinese rhesus macaques (Macaca mulatta; body weight, 3.6–5.2 kg) in the present study. The Animal Care and Use Committee of the Centers for Disease Control and Prevention approved all procedures outlined in this study.

**Virus stocks and inoculation.** SHIVSF162P3 (SIVmac239 backbone and an HIV-1 subtype B, C-C chemokine receptor 5–using envelope) was obtained from the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program (catalog #6526). This virus stock was diluted with RPMI 1040 to 10 TCID/mL for intrarectal inoculations. All virus exposures involved nontraumatic inoculation of 1 mL of SHIVSF162P3 into the rectal vault via a sterile gastric feeding tube. Anesthetized macaques remained recumbent for at least 15 min after each intrarectal inoculation. CM240 (HIV-1 CRF01_AE strain) was obtained from the NIH AIDS Research and Reference Reagent Program (catalog #7703).

**TDF.** TDF, or 9-[(R)-2-[[bis[[isopropoxycarbonyl]oxy]methoxy]phosphinyl]methoxy]propyl] adenine fumarate (1:1), was obtained from Gilead Sciences. TDF (22 mg/kg body weight) was mixed with peanut butter and jelly or was placed in a fruit just before the food was given to the macaques. The animals were monitored, and they usually consumed the food containing TDF within 15–20 min.

**Study design.** Twelve adult, male, Chinese rhesus macaques were divided into 3 groups of 4 animals each. The animals in the control group did not receive TDF; the animals in the daily-TDF group received TDF once daily, and animals in the weekly-TDF group received TDF once weekly. All animals received...
weekly intrarectal inoculations with 10 TCID₅₀ (3.8 × 10⁵ viral RNA copies) of SHIVsf259, with the treated animals receiving the inoculations 2 h after ingestion of TDF. Macaques received TDF even after they became infected. TDF was discontinued after week 36 of the study. The inoculations were stopped once a macaque became infected; macaques that remained uninfected received a total of 14 weekly inoculations. The macaques were monitored for a total of 37 weeks.

**Real-time polymerase chain reaction (PCR) viral load assay.** An SIV gag gene, PCR-based viral load assay was developed for the measurement of SHIV RNA in plasma. A total of 1 mL of plasma underwent ultracentrifugation at 100,000 g for 30 min at 4°C. We removed 800 μL of the supernatant and transferred 200 μL of the pelleted virus to 0.9-mL NucliSens lysis tubes (bioMérieux). Before RNA extraction, we added 3 × 10⁵ virus particles of CM240 (as an external normalizer) to each NucliSens tube, along with the previously added plasma-derived RNA pellet. RNA was extracted using the procedure recommended by the manufacturer (bioMérieux). A standard curve was generated for each viral load assay using SIVmac251; RNA from the SIVmac251 dilution series was extracted along with the external normalizer.

The primers and probe (with SIVmac239 genome coordinates) used to amplify a 67-bp region of SIV were forward primer SIVp15f1 (1256–1278) 5'-GGCAACAGGCTCAGAAAATTTAA-3', reverse primer SIVp15r1 (1348–1323) 5'-TCTCGACTGTGTTTCATTTTCCTTC-3', and probe SIVp15P (1281–1313) 5'-joe AGCCTTFTATATACGTGCATCTGCT-3'. The primers and probe (with HXB2 coordinates) used for amplification of the exogenous control CM240 included forward primer envE2f (6928–6949) 5'-GGACAGGCGCATGTTAAAAAGAGT-3', reverse primer 5'-ENVe2r (7027–7002) 5'-CTCTCTGCTAGACTGCGCATTTAACAG-3', and probe envEP (6965–6977) 5'-fam CACACATGGAATTAAGCCAGTGRTATCTCA-3', and probe SIVp15P (1281–1313) 5'-joe AGCCTTFTATATACGTGCATCTGCT-3'. Reverse transcriptions of 10 μL of RNA in 50-μL reactions were performed using the 2-step TaqMan Gold reverse-transcriptase (RT)–PCR kit (Applied Biosystems) according to the manufacturer’s instructions. We used 10 μL of the cDNA in 50-μL PCR reactions, by use of the 2-step TaqMan Gold RT-PCR kit reagents. PCR was performed using the ABI 7000 Gene Detection System (Applied Biosystems).

**Real-time PCR detection of low-frequency K65R mutant SIV.** The general methods for sensitive real-time PCR-based testing for HIV-1 drug resistance have been described elsewhere [23].

**Sequence analysis of K65R and compensatory mutations.** We amplified 551 bp of the SHIVsf259 RT gene from cell-free virus RNA, using the following nested set of primers (coordinates relative to SMM239): primary primers RTL1f (2980–3001) 5'-GATGGTCAGTGGAGGACAAC-3' and RTL1r (3555–3575) 5'-GGAATTTCCATTTGTGGGAGCT-3' and nested primers RTL2f (3003–3023) CCCCGACCAATCCATACAACA-3' and RTL2r (3532–3553) 5'-GATGGTCAGTGGAGGACAAC-3'. Sequencing was performed with the CEQ (Beckman Coulter), and sequence analysis was performed with Sequencher software (version 4.0.5; Gene Codes).

**Virus-specific antibody responses and lymphocyte phenotype analysis.** Virus-specific serologic responses (IgG and IgM) were measured using methods described elsewhere [19].

**Plasma levels of tenofovir.** An ultrafiltration technique was used to recover tenofovir from plasma. In brief, 500 μL of plasma were transferred to a Microsep 3K Omega Centrifugal Device (Pall Life Sciences) and underwent centrifugation at 6000 g in an Eppendorf 5416 for 60 min. The recovered concentrate was then transferred to autoinjector vials for high-performance liquid chromatography analysis with UV detection by use of external calibration [24]. The lower limit of quantification was 10 ng/mL. The analytical method for detection of tenofovir in plasma was linear over the range of 10–10,000 ng/mL.

**Statistical methods.** The exact log-rank test was used for a discrete-time survival analysis of the treatment and control groups, with use of the number of inoculations as the time variable. A linear mixed-effects regression model was fit to the weekly viral load data for the infected macaques, with use of the macaques as the random effect and the 3 study groups as the fixed effects. In addition, 2 nonparametric tests, the Wilcoxon rank sum test was used to analyze the peak viral load of the infected macaques. Inferences regarding the per-exposure effect of tenofovir were based on a discrete-time transmission probability model that assumed that the probability of infection is independent of the number of prior exposures. Maximum-likelihood estimates were obtained using standard methods. Power calculations were conducted using a simulation study. All statistical analyses for calculation of the efficacy of tenofovir were performed using SAS software (version 9.1; SAS Institute) and StatXact software (version 6.3; Cytel).

**RESULTS**

**Plasma levels of tenofovir.** Before starting the virus challenge study, we conducted a time-course analysis of plasma levels of tenofovir in one SIV-naïve macaque. Blood samples were collected at various intervals after oral administration of TDF with food. Levels of tenofovir in blood reached a peak (633 ng/mL) 2 h after TDF administration and then began to decrease, and, by 72 h after administration, TDF was undetectable in plasma (data not shown). The area under the time-concentration curve was 3967 ng × h/mL. Previously published data have also described peak plasma levels of tenofovir achieved 1–2 h after oral TDF administration [27]. Therefore, in the present
study, the macaques in the 2 treatment groups were inoculated with SHIV<sub>SF162P3</sub> 2 h after ingestion of TDF with food.

During the study, the plasma levels of tenofovir in all 3 groups of animals were measured in blood samples collected 2 h after oral TDF administration at various study weeks. For a total of 34 blood samples, the mean and median tenofovir levels were 2003 ng/mL (SD, ± 3521) and 499 ng/mL (range, 10–4376 ng/mL), respectively. When the blood samples were categorized according to the treatment group of the macaques from which they were obtained (18 samples were obtained from the group receiving treatment daily and 16 samples were obtained from the group receiving treatment weekly), the median levels of tenofovir were similar for each group: 537 ng/mL (range, 10–3308 ng/mL) and 422 ng/mL (range, 19–14,376 ng/mL), respectively (data not shown). There was no statistical difference in tenofovir levels between the group receiving TDF daily and the group receiving TDF weekly. For 5 of 8 TDF-treated macaques, tenofovir levels were measured in blood samples collected at the time of the virus exposure that resulted in systemic infection. In 4 of these 5 macaques, the tenofovir levels were low (29, 10, 69, and 137 ng/mL) at the time of the virus inoculation that resulted in systemic infection, whereas, in the fifth macaque, the tenofovir level was high (1854 ng/mL).

**Systemic infection resulting from repeated intrarectal inoculations with SHIV<sub>SF162P3</sub>** To ascertain the presence of systemic infection, we used detection of cell-free viral RNA in blood as the initial marker. Infection was confirmed by the detection of cell-associated SHIV<sub>SF162P3</sub> provirus DNA, which was generally detected during the same week when plasma cell-free virus was detected (provirus data not shown). Prophylaxis with TDF increased the median time to infection for the groups receiving treatment daily and weekly (6.0 weeks and 7.0 weeks, respectively), compared with that noted for the control group (1.5 weeks) (figure 1). One macaque (RQ4180) in the daily-TDF group remained uninfected after receiving 14 inoculations with virus (figure 1). Survival analysis showed no statistical difference between the treated and control groups at 14 weeks (\(P = .315\), by exact log-rank test). To determine whether macaque RQ4180, which had remained virus free after 14 weekly challenges, was susceptible to SHIV infection in the absence of TDF treatment, the animal was rested for 30 weeks, during which time no virus challenge or TDF was administered. After the rest period, weekly intrarectal SHIV inoculations were resumed in the absence of daily TDF treatment; the animal became infected after 2 inoculations.

Using a discrete-time transmission probability model, we estimated that oral TDF prophylaxis of macaques in our study resulted in a 60% decrease in the per-exposure probability of infection (95% confidence interval, −0.36 to 0.86). This result did not achieve statistical significance \((P = .13)\). However, with a total sample size of 12 animals, the present study was not powered to detect effects of this size.

**Cell-free virus loads in plasma.** In most of the control and treated animals, peak viral levels were reached within 1–2 weeks after the first detection of virus RNA in plasma (figure 2). The differences in peak viral loads between the control and daily-TDF groups and between the control and weekly-TDF groups were not statistically significant as measured by the Wilcoxon
Figure 2. Cell-free plasma viral loads and virus-specific antibody responses (IgG and IgM) in plasma. Viral loads are shown for control macaques (A), macaques receiving tenofovir disoproxil fumarate daily (daily-TDF) (C), and macaques receiving TDF weekly (weekly-TDF) (E). The horizontal dotted line denotes the assigned cutoff value (500 copies/mL) for the assay. The sensitivity of the assay was 100 copies/mL. B, D, and F, Virus-specific antibody responses in plasma. The optical density cutoff value is denoted by a dashed line on the graph. Nos. in parentheses denote the no. of weekly simian human immunodeficiency virus challenges after which each macaque became infected. OD$_{450}$, optical density measurement at 450 nm.

rank sum test ($P = .23$ and $P = .34$, respectively). The median peak viral loads in the control, daily-TDF, and weekly-TDF groups were 6.3, 6.0, and 7.6 log$_{10}$ copies/mL, respectively. Although there was a 1.6-log$_{10}$ difference between the median peak viral loads in the daily-TDF and weekly-TDF groups (the largest value in the daily-TDF group was smaller than the smallest value in the weekly-TDF group), this difference was not statistically significant ($P = .06$). It is of interest to note that the peak viral load in macaque RQ4115, which was in the daily-TDF group, was $\sim$2 log$_{10}$ copies lower than the peak viral loads in the remaining 2 infected macaques that were part of the daily-TDF group (fig. 2). For each of the 3 groups, statistical evaluation of the changes in viral loads at consecutive time points after infection indicated that there was no difference in viral load trajectories between infected control animals and infected tenofovir recipients (data not shown).

Virus-specific antibody responses. In the control animals, virus-specific antibody responses were detected 3–5 weeks after
the firs detection of virus in plasma (fig 2). Similar casts were noted in the daily-TDF group (3–7 weeks) and weekly-TDF group (3–5 weeks); however, no antibody responses were observed for macaque RQ4463 (fig 2), and antibody responses in macaque RQ4115 were very low throughout the course of the study.

Emergence of the tenofovir-resistant K65R mutation and compensatory mutations. The K65R mutation was not detected through 31 weeks of the study (data not shown). In addition, sequencing analysis of the RT gene did not detect any of the known compensatory mutations (K64R, N69S/T, I118V, and S211N) [28].

DISCUSSION

The present study represents the firs application of a macaque model of repeated exposures to virus in the assessment of antiretroviral prophylaxis for the prevention of SHIV transmission via rectal exposure. These data demonstrate that treatment with oral TDF protected against 14 virus exposures in one macaque and delayed infection in the other macaques; however, the overall differences were not statistically significant at week 14 of the study (P = .315). That we were subsequently (after a significant drug-washout time) able to infect the macaque that did not become infected after 14 virus challenges suggests that oral TDF must have played a role in preventing infection in this macaque. The delays in infection were similar for macaques that received oral TDF daily and macaques that received oral TDF weekly, suggesting that the time interval (2 h in this study) between virus exposure and TDF dosing is important for protection; it is unknown what the outcome would be if the exposure occurred several hours or days after TDF dosing. In the present study, macaques received the equivalent of the human therapeutic dose of TDF; however, the virus-challenge levels, although more physiologic than those in other high-dose models of infection, were ~5-fold higher than the levels noted in human semen. This higher virus dose may have also contributed to the partial efficacy observed. On the basis of decreased per-exposure probability of infection in macaques and the observation of partial protection, we believe that interventions with oral TDF may reduce rectal transmission of HIV. However, optimism must be tempered because, as the macaque data indicate, protection may not be absolute against repeated high-risk virus exposures.

Although the firs infections did not occur until after 6 challenges, it is possible that there may have been low-level infection during the observed 5-week “protection window.” Although low-level infection was not detected in circulating peripheral blood mononuclear cells (data not shown), we cannot rule out infection in other tissue sites, such as the lymph nodes and the gut, that subsequently disseminated systemically, perhaps during a period of lower-than-optimal drug levels. Periods of transient viremia before persistent viremia have been documented for macaques exposed to successive low-dose challenges with SIVmac251 [29]; however, we did not observe such transient infections in our macaques infected with SHIVSF162P3.

Why was infection not blocked completely? We used an antiretroviral drug that targets the virus after it has entered target cells. To provide complete protection, the drug must reach all target cells within the mucosal tissues, and levels of the drug within every target cell must reach maximum levels of efficac to block virus reverse transcription. It is likely that, in the face of repeated virus challenges, the virus may break through during a period of lower drug levels (a drug trough). Our results showed that, although the median plasma levels of tenofovir in TDF-treated macaques were similar to the median levels in humans receiving the US Food and Drug Administration-approved 300-mg daily dose for the treatment of HIV-1 infection [27], tenofovir levels in macaque plasma were variable; however, such variability was also noted in humans [27]. Plasma levels of tenofovir are higher and more consistent when given intravenously to humans [30] or subcutaneously to macaques (W. Heneine, personal communication). Therefore, the TDF dose used as standard antiretroviral therapy may be lower than the dose necessary to block infection (reverse transcription) in all target cells.

There is concern that seroconversion may be delayed after infection in humans who may receive TDF chemoprophylaxis, because of TDF-mediated suppression of viral replication resulting in the presence of subantigenic doses of virus. In such a situation, infection may be missed by first-lin tests for the detection of HIV antibody. In the present study, we did not observe any significant delays in the development of SHIV-specifi antibody responses in TDF-treated macaques. An exception was macaque RQ4463, which was in the weekly-TDF group and did not develop any detectable antibody responses. In this macaque, the lack of an antibody response was associated with high plasma viremia throughout the course of the study. We do not know whether the lack of SHIV-specifi antibody responses was driven by tenofovir or host/genetic-related factors. It is known that ~25% of rhesus macaques do not sero-convert after SIV infection and progress to death with high virus loads [31–33].

There exists no precedent for giving chemoprophylaxis to large populations of people at risk for infection through repeated exposure to HIV. Only well-planned clinical trials involving humans will directly answer whether antiretroviral chemoprophylaxis will protect against HIV infection. The Centers for Disease Control and Prevention is sponsoring 3 clinical trials of TDF [34]. Two of these trials are efficac trials involving heterosexuals in Botswana and injection drug users in Thailand. The third trial is an extended safety trial conducted in the United States and involving men who have sex with men. In
addition, the NIH is sponsoring an efficac trial of TDF in Peru among men who have sex with men. Although caution must be used when extrapolating results for macaques to humans, our data suggest that TDF might delay infection and that this could, in turn, reduce overall rates of transmission of HIV. We also cannot extrapolate from these results of a rectal transmission study to estimate the efficac of TDF for vaginal or parenteral HIV exposures. The physiological prof of transmission differs according to the route of exposure, and it appears that the risk for infection is greater when exposure is rectal rather than when it is vaginal [35]. In addition, we did not observe the emergence of tenofovir resistance mutations in the virus even after prolonged exposure to the drug. Given the current lack of an effective biomedical intervention, tenofovir prophylaxis may be of benefit More important, emphasis on the use of protective measures, such as abstinence, condoms, and mutual monogamy with an uninfected partner, should remain unreleenting.

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