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Inefficient Vaginal Transmission of Tenofovir-Resistant HIV-1

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Transmission of drug-resistant HIV has been postulated to be a threat to current first-line antiretroviral therapy (ART) regimens and the efficacy of several antiretroviral-based preexposure prophylaxis (PrEP) strategies being tested. Here we evaluated the effect of the common tenofovir (TFV) resistance mutation K65R on vaginal HIV transmission. Our results demonstrate that despite no overt loss of overall replication competence *in vivo*, this mutation results in significantly reduced mucosal transmission. When transmitted, the mutant virus eventually reverted to the wild type in 2 of 3 animals examined.

In the absence of a cure or vaccine, and despite valuable efforts toward better human immunodeficiency virus (HIV) education, including safe sex practices, the HIV epidemic continues to grow at a faster pace than the current availability of antiretroviral therapy (ART). For every two people who begin ART, five are newly infected (1). Of the people infected, only 47% have access to ART in low- and middle-income countries (2). There is a great need to prevent transmission of HIV. To address this need, extensive efforts are being made to develop and implement effective preexposure prophylaxis (PrEP) approaches. So far, the greatest progress has been made using antiretroviral drug-based treatment as prevention and PrEP (3, 4). When the patient has a positive diagnosis and access to a full ART regime under a doctor's guidance, early treatment is exceedingly effective for preventing transmission of HIV to uninfected partners (4). Unfortunately, a significant number of HIV-positive individuals do not know their infection status, especially during acute infection when transmission potential is highest, increasing the need for alternatives such as PrEP. Most current PrEP clinical trials are investigating the use of antiretroviral drugs either singularly or as a two-drug combination for systemic or topical use (3, 5–8). This raises an important concern with the dual use of antiretroviral drugs for both treatment and prevention: the consequences of the development and transmission of drug-resistant HIV.

HIV-1 develops resistance to virtually all drugs currently available for treatment (9, 10). For this reason, current ART therapies consist of a cocktail of multiple drugs with different classes of action to prevent or at least postpone the development of drug-resistant HIV within the patient's life span. Drug-resistant viruses can be transmitted (11–14). During new infections, certain mutations like M184V are rarely detected by routine genotyping, but significantly higher proportions can be detected using more-specific methodology (11, 13, 14). The inherent ability of replicating HIV to revert to a drug-sensitive genotype in the absence of drug pressure makes it difficult to study in patients, especially if (i) the time, duration, and route of infection are unknown, (ii) there is no way to prove ART-naïve status, and (iii) the HIV sequence in the infecting partner is unknown. Despite these difficulties, genotypic analysis of ART-naïve patients has provided evidence that drug-resistant HIV-1 is being transmitted and can result in treatment failure (15–21). Given that animal studies are the best option to overcome the inherent limitations of human studies (22), we utilized humanized mice to investigate *in vivo* transmission of drug-resistant HIV-1.

Tenofovir (TFV) is the drug most commonly used in clinical trials evaluating systemic and topical PrEP. Tenofovir disoproxil fumarate, the oral formulation of TFV, is also part of every DHHS-recommended first-line therapy (23). For this reason, we chose to study transmission of tenofovir-resistant HIV. The mutation of the lysine at amino acid position 65 in HIV reverse transcriptase to an arginine (K65R) confers resistance to tenofovir as well as other nucleoside reverse transcriptase inhibitors (NRTIs). For this reason, K65R is on both the WHO and International AIDS Society (IAS) surveillance lists for HIV genotyping (9, 10). There is clinical evidence that HIV containing the K65R mutation can be transmitted after mucosal exposure, albeit at a lower frequency than other mutations, like M184V (11, 13, 15, 20). To evaluate the role of this single amino acid mutation on mucosal HIV transmission, we introduced the K65R mutation (AAA to AGA) into a proviral clone of HIV-$1_{JR\text{-}CSF}$ (24). In addition, to differentiate the mutant virus from the parental clone after reversion, a second, silent mutation (TAT to TAC; tyrosine) was included to act as a molecular marker.

To confirm a decrease in the susceptibility of the mutant virus to TFV, we determined the *in vitro* 50% inhibitory concentration (IC_{50}) for wild-type $HIV_{JR\text{-}CSF}$ and the isogenic mutant $HIV_{JR\text{-}CSF\ K65R}$. The K65R mutation conferred a 4.7-fold increase in the *in vitro* IC_{50} for TFV, a change which is comparable to the 2- to 4-fold reduction in susceptibility reported previously (25, 26) (Fig. 1). Previous *in vitro* studies have shown that the K65R mutation reduces the function of viral reverse transcriptase (26, 27). It is unknown to what extent this defect affects viral replication *in vivo*. To test the *in vivo* replication capacity of $HIV_{JR\text{-}CSF\ K65R}$, humanized mice (28, 29) were inoculated via intraperitoneal (i.p.) injection of 3×10^4 tissue culture infectious units (TCIU), and viral load in plasma was monitored over time (30). Longitudinal analysis of plasma viral load showed no difference in the *in vivo* replication of the K65R mutant and wild-type strains (Fig. 2) in this group of five animals, suggesting that there are not large differences in the *in vivo* fitness of the mutant virus. Sequence analysis of plasma virus RNA from HIV-$1_{JR\text{-}CSF\ K65R}$-infected mice confirmed the presence of the K65R mutation 2 weeks postinfection. However, subsequent time points showed a population of wild-type virus. Se-

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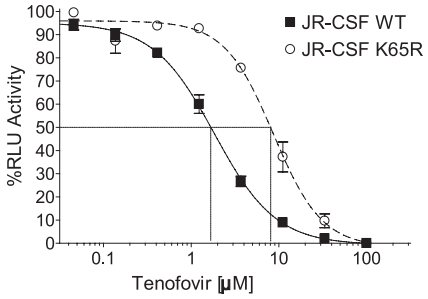

FIG 1 Introduction of the K65R mutation into $HIV_{JR\text{-}CSF}$ results in a 4.7-fold increase in *in vitro* IC_{50} using a luciferase-based assay in TZM-bl indicator cells. Serial dilutions of tenofovir were applied to indicator cells in triplicate and allowed to incubate for 30 min before an equal number of tissue culture infectious units (TCIU) of either wild-type or mutant virus was applied to all wells. Two days later, the medium was removed, ONE-Glo reagent (Promega) was added, and the amount of luciferase activity was measured. Each curve was normalized to wells infected with that specific virus (wild-type or K65R virus) in the absence of the drug. RLU, relative light units.

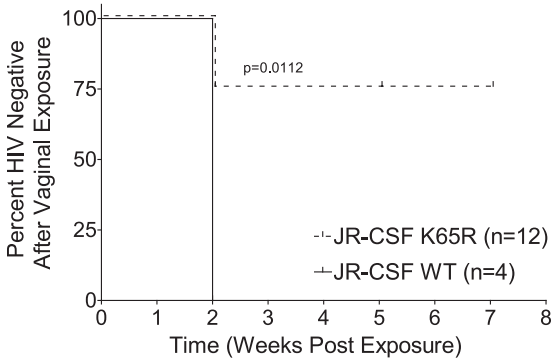

FIG 3 The K65R mutation reduces vaginal transmission efficiency of HIV-1 by 75%. Humanized BLT mice were prepared and validated as previously described (30, 31, 38). Mice were exposed vaginally to a single dose of $HIV\text{-}1_{JR\text{-}CSF}$ or $HIV\text{-}1_{JR\text{-}CSF\ K65R}$ (3.5×10^5 TCIU). Infection was monitored as a function of viral load in plasma. The Kaplan-Meyer plot shows the percentage of HIV-negative mice as a function of the number of weeks postexposure until the first peripheral blood HIV-1 detection. In 3/12 (25%) mice, viral load was readily detectable 2 weeks postexposure. In 9/12 (75%) mice exposed to the K65R mutant, no viral load was detected at any time point analyzed and no viral DNA was found in tissues at harvest, confirming the lack of transmission.

quence analysis indicated that reversion of the K65R mutation was always to the original sequence. It should be noted that the molecular marker, present only in the mutant virus, served to exclude the possibility of contamination with wild-type virus.

Having demonstrated the replication capacity of the K65R mutant virus *in vivo*, we next evaluated its capacity to transmit mucosally. For this purpose, we utilized BLT humanized mice (30). The female reproductive tract of BLT mice is reconstituted with all the cells relevant for HIV transmission, including human T cells, monocyte/macrophages, and dendritic cells (30, 31). BLT mice were vaginally exposed once to equal infectious doses of wild-type $HIV\text{-}1_{JR\text{-}CSF}$ or the isogenic K65R mutant virus (3.5×10^5 TCIU). Three independent exposures ($n = 4$) were performed on three different dates. The results of these vaginal exposures showed a dramatic decrease in the transmission efficiency of the K65R mutant virus (Fig. 3). Specifically, whereas all the mice exposed to the wild-type virus were infected (4/4), only 25% of the mice exposed to the mutant virus were infected (3/12). This difference in vaginal HIV transmission was highly statistically significant by log rank analysis ($P = 0.011$; Mantel-Cox). These results demonstrate that the K65R mutant is vaginally transmitted at a greatly reduced rate compared to that of the wild-type virus. Interestingly, these results seem at odds with those recently published by Cong et al. (22) using simian immunodeficiency virus (SIV). However, these results may be due to the facts that a different mutation was used and that additional fitness compensatory mutations were introduced into the provirus used by Cong et al. (22).

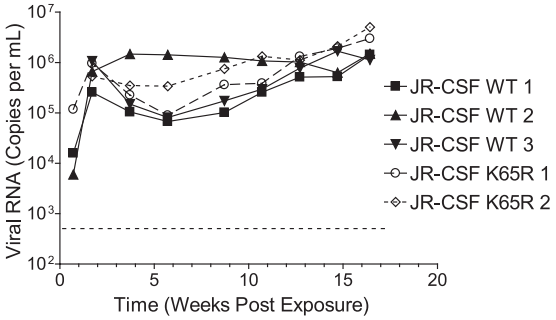

FIG 2 *In vivo* replication of $HIV_{JR\text{-}CSF}$ and $HIV_{JR\text{-}CSF\ K65R}$ after i.p. injection into humanized mice shows no overt difference in replication capacity. Humanized NOD/SCID/$\gamma^{-/-}$ mice (28, 29) were infected with equal amounts of either $HIV\text{-}1_{JR\text{-}CSF}$ or $HIV\text{-}1_{JR\text{-}CSF\ K65R}$ (3×10^4 TCIU) by i.p. injection. The course of infection was monitored by determining plasma viral loads. Dotted line indicates the limit of detection of the assay.

To determine if the transmitted virus contained the K65R mutation, plasma viral RNA was sequenced at different times after exposure. Four weeks postexposure, we noted the presence of only mutant virus in one mouse (M1), the presence of only wild-type (reverted) virus in a second mouse (M2), and the presence of both mutant virus and wild-type (reverted) virus populations in a third mouse (M3). Longitudinal analysis of the virus found in the plasma of one of the infected mice (M3) showed the presence of both mutant and wild-type viruses at weeks 4 and 6 postinfection and the presence of wild-type virus at all subsequent time points (Table 1). Cervicovaginal lavage (CVL) fluid from this mouse also showed the presence of both wild-type and mutant virus 4 weeks postinfection. Subsequently, only the wild-type virus was found in the CVL fluid (Table 1). Analysis of the virus present in the different tissues from two of the infected mice generally reflected what was observed in the periphery. However, in one mouse, the mutant virus was found in the plasma but all tissues analyzed contained both the wild-type and mutant viruses. Interestingly, analysis of the virus present in tissues 14 weeks postinfection showed the wild-type virus in all tissues except the thymic organoid, in which both drug-resistant and wild-type viruses were found (Table 1). These results are consistent with the hypothesis of Weinberg et al. suggesting that transmitted viruses that contain reversible mutations become archived in lymphocyte reservoirs (14).

In summary, the topical or systemic use of antiretroviral drugs for the purpose of preventing HIV acquisition has the potential to curtail the spread of AIDS, and some PrEP strategies have shown great promise (4, 5, 7, 32, 33). The fact that tenofovir is a successful first-line drug for the treatment of HIV infection has made this compound the drug of choice for most prevention trials (34). However, this dual-use approach is not without risk, as there is significant potential to expand the pool of drug resistance in com-

TABLE 1 Sequence analysis demonstrates reversion of the K65R mutation over time in peripheral blood, cervicovaginal lavage fluid, and tissues of infected BLT mice[a]

Mouse	Wk postexposure	Amino acid(s) at position 65 in sample from:					
		Peripheral blood	Vaginal lavage fluid	FRT	Lymph node	Organoid implant	Lung
M1	4	R only	NA	NA	K and R	K and R	K and R
M2	4	K only	NA	NA	K only	K only	K only
M3	4	K and R	K and R				
	6	K and R	K only				
	9	K only	K only				
	13	K only	K only				
	14	K only	K only	K only	K only	K and R	K only

[a] Bone marrow/liver/thymus mice were exposed once intravaginally to the mutant virus. Infection was monitored in plasma by determining the viral load. Two mice were harvested 4 weeks postinfection (M1 and M2), and one was harvested 14 weeks postexposure (M3). Peripheral blood and vaginal lavage fluid samples from this mouse were collected longitudinally. FRT, female reproductive tract; K, lysine; R, arginine; NA, not available. PCR primer sets used to amplify the reverse transcriptase (RT) are as follows: outer/first reaction, 5′-GCTCTATTAGATACAGGAGC-3′ and 5′-CCTAATGCATATTGTGAGTCTG-3′; inner/second reaction, 5′-GTAGGACCTACACCTGTCAAC-3′ and 5′-CCTGCAAA GCTAGGTGAATTGC-3′. Amplification products were sequenced in bulk.

munities utilizing PrEP (32, 35). Here we tested K65R-mutated HIV-1 in humanized mice and found that, as in humans, the HIV carrying the K65R mutation (i) is replication competent (Fig. 2), (ii) is present in cervicovaginal secretions (Table 1), and (iii) reverts to the wild type in the absence of drug selection although the mutant virus remains detectable (Table 1). Finally, we tested the ability of K65R mutant HIV to transmit vaginally and found that it can transmit, albeit at a significantly lower efficiency than that for the wild type (Fig. 3). At this point, the molecular basis for this lower transmission is not known. However, analysis of the K65R mutant has shown that it has a decreased replication capacity compared to the wild type in several *in vitro* model systems (36, 37). Overall, our results demonstrate that if this tenofovir-resistant virus is present in the transmitting partner, there is the potential for the mutant virus to be transmitted to the uninfected partner with lower efficiency than wild-type HIV-1.

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