Selection of a Simian-Human Immunodeficiency Virus Strain Resistant to a Vaginal Microbicide in Macaques[†]

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PSC-RANTES binds to CCR5, inhibits human immunodeficiency virus type 1 (HIV-1) entry, and has been shown as a vaginal microbicide to protect rhesus macaques from a simian-human immunodeficiency virus chimera (SHIV_{SF162-p3}) infection in a dose-dependent manner. In this study, *env* gene sequences from SHIV_{SF162-p3}-infected rhesus macaques treated with PSC-RANTES were analyzed for possible drug escape variants. Two specific mutations located in the V3 region of gp120 (K315R) and C-helical domain of gp41 (N640D) were identified in a macaque (m584) pretreated with a 100 μ M dose of PSC-RANTES. These two *env* mutations were found throughout infection (through week 77) but were found at only low frequencies in the inoculating SHIV_{SF162-p3} stock and in the other SHIV_{SF162-p3}-infected macaques. HIV-1 *env* genes from macaque m584 (*env*_{m584}) and from inoculating SHIV_{SF162-p3} (*env*_{p3}) were cloned into an HIV-1 backbone. Increases in 50% inhibitory concentrations to PSC-RANTES with *env*_{m584} were modest (sevenfold) and most pronounced in cells expressing rhesus macaque CCR5 as compared to human CCR5. Nonetheless, virus harboring *env*_{m584}, unlike inoculating virus *env*_{p3}, could replicate even at the highest tissue culture PSC-RANTES concentrations (100 nM). Dual-virus competitions revealed a dramatic increase in fitness of chimeric virus containing *env*_{m584} (K315R/N640D) over that containing *env*_{p3}, but again, only in rhesus CCR5-expressing cells. This study is the first to describe the immediate selection and infection of a drug-resistant SHIV variant in the face of a protective vaginal microbicide, PSC-RANTES. This rhesus CCR5-specific/PSC-RANTES resistance selection is particularly alarming given the relative homogeneity of the SHIV_{SF162-p3} stock compared to the potential exposure to a heterogeneous HIV-1 population in human transmission.

In light of recent failures of promising human immunodeficiency virus type 1 (HIV-1) vaccine candidates, microbicides have regained prominence as a method to prevent HIV-1 transmission. Unfortunately, vaginal microbicide trials with well-tolerated antimicrobial compounds such as carrageenan (Carraguard) were ineffective at blocking HIV-1 transmission (31, 36). The focus in microbicide studies has now shifted to specific HIV-1 inhibitors that have proven effective at blocking primate lentivirus transmission in macaque animal models (21, 38). In particular, HIV-1 entry inhibitors are promising microbicide candidates that can block virus replication prior to stable HIV-1 integration into host cell DNA (16).

PSC-RANTES, a nonanoyl N-terminally modified derivative of the natural chemokine RANTES targets the CCR5 coreceptor, is one of the most potent entry inhibitors (30) and is a proven vaginal microbicide in its ability to protect macaques from a vaginal challenge with a simian immunodeficiency virus (SIV)-HIV chimeric virus, SHIV_{SF162-p3}, containing the HIV-1 envelope (*env*) gene (21). PSC-RANTES and other RANTES

analogues have been shown to utilize two different mechanisms to prevent HIV-1 infection by binding to CCR5 (27). First, PSC-RANTES induces CCR5 internalization and sequestration inside the cell (27) or can act as a competitive inhibitor of HIV-1 entry (22). As a consequence of the latter, diverse primary HIV-1 isolates display variable susceptibility to PSC-RANTES inhibition similar to other CCR5 antagonist inhibitors such as TAK-779 and Viroviroc (28, 32).

PSC-RANTES administered at a high dose (1 mM) was shown to be 100% protective against vaginal SHIV_{SF162-p3} (HIV-1_{SF162} Env in an SIV backbone) challenge in rhesus macaques treated with progesterone (21). Reasons for the dose-dependent decrease in protection (i.e., <1 mM) are likely due to a complex set of host, viral, and drug interactions. This study, however, focuses on the possible selection of PSC-RANTES resistance among SHIV_{SF162-p3} variants during transmission and/or infection. Selection of drug resistance has been described in macaques infected with SIV or SHIV through intravenous challenge and treatment with various reverse transcriptase inhibitors (e.g., tenofovir) (1). In these cases, drug-resistant variants likely emerged from the infecting SHIV or SIV population by a mechanism similar to that described during suboptimal antiretroviral treatment of HIV-infected humans. However, drug resistance has yet to be identified in macaque microbicide studies involving the systemic, intravenous, or vaginal administration of anti-HIV-1 drugs. Several human trials with anti-HIV-1 drugs used as microbi-

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cides are under way or in development (7, 20, 29). These trials provide a strong rationale for thorough screening of drug resistance in the current macaque models. Nonetheless, prevailing opinion and recent models suggest that resistance will not present significant problems unless the drug is systemically absorbed and is ineffective at blocking transmission (39).

Human transmission of HIV-1 likely involves a transfer of a diverse virus population (up to 10% in nucleotide diversity), and yet, infection is established in the recipient by very few HIV-1 variants (15, 40). Diversity in the transferred virus population may pose problems, as illustrated in prevention of mother-to-infant transmission. Although nevirapine is ~80% effective in blocking perinatal HIV-1 transmission (33), at least 50% of infants harbor nevirapine-resistant virus within 48 h of delivery and nevirapine treatment (3, 35). Given the relative homogeneity of the inoculating SHIV in macaque studies versus inoculating HIV-1 in human transmissions, current macaque microbicide models may underestimate the possible selection of drug-resistant HIV-1 from the inoculating SHIV. In this study, we investigated the possible selection of PSC-RANTES-resistant virus in macaques vaginally pretreated with PSC-RANTES and then challenged with SHIV_{SF162-p3}. We analyzed the entire *env* genes from 59 SHIV_{SF162-p3} viruses taken from multiple plasma samples of 14 infected rhesus macaques pretreated with various doses of PSC-RANTES (ranging from 1 μ M to 1 mM). In one macaque (m584) vaginally treated with a partially protective dose of PSC-RANTES (100 μ M), two distinct but linked amino acid Env substitutions (K315R and N640D) were present in the SHIV_{SF162-p3} population at the earliest sample time and throughout the 77 days of infection. The following experiments determined how these mutations (in the autologous sequence) may impact resistance to PSC-RANTES, fitness, and utilization of the rhesus macaque (Rh-CCR5) versus human CCR5 (Hu-CCR5) coreceptor.

MATERIALS AND METHODS

Cells and viruses. U87.CD4.CCR5 human glioma cells (U87.CD4.Hu-R5) were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (8). U87 and 293T cells were maintained as previously described (22). U87.CD4 cells expressing Rh-CCR5 were created as described below. Viruses were propagated on U87.CD4.Hu-R5 cells, and titers were determined on both U87.CD4 cell lines expressing either Hu-CCR5 or Rh-CCR5 by the Reed-Muench end point titration method. Virus production was measured with a reverse transcription (RT) assay utilizing radiolabeled dTTP ([α -³²P]TTP) (6). Construction of chimeric viruses containing the envelope genes from SHIV viruses and the outline of how we analyzed them in this paper are depicted in schematic form in Fig. S2 in the supplemental material.

Production of the U87.CD4.Rh-R5 cell line. U87.CD4 Rh-CCR5 (U87.CD4.Rh-R5) cells were created by introducing pBABE.Rh-CCR5 (obtained from the NIH AIDS Research and Reference Reagent Program from Preston Marx) into U87.CD4 cells (obtained from the NIH AIDS Research and Reference Reagent Program from Hongkui Deng and Dan R. Littman) using a lentiviral vector packaging system (vectors pMD.g and pCMV Δ R8.9I) (8, 10, 41). Following initial selection of this cell line, CCR5-positive cells were sorted (as described below) and individual cells were propagated. CCR5 expression on the surface of these propagated clones was measured by flow cytometry using the 3A9 antibody (see below), and cells maintaining high levels of expression were used in subsequent experiments.

Sequence analysis of SHIV_{SF162-p3} viruses from rhesus macaque plasma. Plasma from rhesus macaques infected with SHIV_{SF162-p3} in the presence of PSC-RANTES were obtained as described previously (21). Viral RNA was extracted from the plasma using a viral RNA minikit (Qiagen) and then reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA) and the primer SHIV ext anti (for primer sequences, see

Table S1 in the supplemental material). Whole *env* sequences were amplified with SHIV ext sense and SHIV ext anti using high-fidelity *Taq* polymerase (Roche). Nested PCR (SHIV nest sense-SHIV nest anti) was then used to further amplify *env*. DNA sequencing was performed by Davis Sequencing Services using the primers SIS2 and SHIV int anti. Sequences were then analyzed using BioEdit software and aligned using Clustal X. The sequences are available under GenBank accession no. FJ768962 to FJ769022.

OLA. The oligonucleotide ligation assay (OLA) was performed as previously described (19). External PCR products derived from reverse-transcribed plasma RNA or cellular proviral DNA (for fitness competitions) was amplified by PCR using the primer set E110-E45A (1,342 bp) to encompass both amino acid changes to be analyzed. For analysis of the mutational prevalence, samples were analyzed from virus extracted at three different time points from macaque m584, from the inoculating SHIV_{SF162-p3} virus population, from a population of virus derived from two macaques treated with either 10 μ M or 33 μ M PSC-RANTES, and from three populations of virus derived from phosphate-buffered saline (PBS)-treated macaques. Control templates were amplified with the same primer set from plasmid clones containing SHIV *env* genes with or without both the K315R and N640D mutations to create standards. Upstream OLA probes were designed to contain either the 315R or 640D mutation at the 3' end and were 5' radiolabeled with [γ -³²P]ATP using T4 polynucleotide kinase (Invitrogen). A primer containing a 5'-triphosphate group was designed to anneal immediately downstream of the nucleotide of interest. The 3' end of the upstream primer anneals to the study template at the site of the mutation, and the downstream primer 5' end anneals to the next nucleotide downstream from the mutation. For each OLA reaction, 5 and 25 ng of the PCR-amplified template from each population described above were mixed with 1.5 pmol of ³²P-radiolabeled upstream primer, 1.5 pmol of the downstream primer, and 2.5 U Ampligase DNA ligase (Epicentre Technologies, Madison, WI) in a 12- μ l reaction mixture containing 16.7 mM Tris-HCl (pH 8.3), 0.07% Triton X-100, 0.8 mM dithiothreitol, 10 mM KCl, 8.3 mM MgCl₂, and 0.83 mM NAD. Reactions were subjected to 30 ligation cycles of 93°C for 30 s and 37°C for 4 min and were stopped by addition of 10 μ l loading buffer (0.1 M EDTA, 0.1% Triton X-100, 50% formamide, bromophenol blue, and xylene cyanol dyes in water). Reaction mixtures (3 μ l) were then separated by 10% denaturing polyacrylamide gel electrophoresis for 2 h and dried. Gels were subjected to autoradiography and phosphorimaging. Bands were quantitated using Quantity One software (Bio-Rad). The percentages of populations containing the K315R and N640D mutations were calculated based on fitting signals from control templates to the following exponential: $M = A_1[\exp(I/t_1)] + y_o$, where (I) is corrected signal intensity, M is template mass input in ng, A_1 defines curve height, t defines curve rise rate, and y_o defines vertical offset using Origin Lab software (OriginLab Corp., Northampton, MA), with a chi-square tolerance fitting of $1E-9$. Mass was calculated from the corrected signal given by 12 ng of negative control template and was subtracted from y_o , serving as a cutoff for subsequent calculations. Two dilutions from each sample were used for each probe (5 ng and 25 ng). The mass calculation from the corrected signal intensity from 5 ng input was multiplied by 5 and compared to the signal from the 25-ng dilution. This is the normalized apparent copy number (C_s), which is specific to each preparation of probe. The larger copy number was then used to calculate the mutant content based on the following equation: % mutant = $100 \times (C \text{ mutant}) / (C \text{ mutant} + C \text{ wild type})$.

Construction of *env* and gp120 chimeric viruses. Briefly, pREC_{Δenv}/URA3 was digested with SacII (Promega, Madison, WI) and transformed with lithium acetate into *Saccharomyces cerevisiae* MYA-906 cells (ATCC) with *env* PCR products. Following yeast homologous recombination and amplification of the plasmids in yeast cells, plasmids were extracted from the yeast and transformed into electrocompetent *Escherichia coli* DH10B cells (Invitrogen) as previously described (24). Bacterial colonies were grown, and the plasmids were then extracted from the clones using Qiagen midiprep kits. The *env* genes from the pRec/SHIV-Env constructs were then digested out of the shuttle vector using EcoRI and XhoI. These *env* genes containing either whole *env* or gp120 from the SHIV viruses were then ligated into plasmid pNL4-3 (previously digested with EcoRI and XhoI to remove the *env* gene) using NEB ligase (New England Biolabs, Inc., Ipswich, MA). Correct insertion of the gp120 or *env* gene into pNL4-3 was verified by sequencing the *env* region of the plasmid after the cloning procedure was finished. This sequence analysis was also used to verify which clones contained the consensus sequence associated with SHIV viruses derived from plasma of different rhesus macaques. Plasmids containing SHIV Env proteins or gp120 were then transfected into 293T packaging cells using Effectene (Qiagen), and the packaged virus was collected 48 h posttransfection. Envelope genes were sequenced following transfection from each virus population, and then the transfection supernatant viruses were used to propagate infectious chimeric viruses on U87.CD4.Hu-CCR5 cells. Again, chimeric viruses were se-

quenced following propagation to verify the presence or absence of mutations. Titers of viruses were determined on both the U87.CD4.Rh-R5 and U87.CD4.Hu-R5 cell lines using the Reed-Muench endpoint titration method. A schematic representation of this procedure is shown in Fig. S2 in the supplemental material.

FACS analysis. Six-well plates containing 300,000 U87.CD4.Hu-CCR5 or U87.CD4.Rh-CCR5 cells were plated and treated the next day with 100 nM PSC-RANTES or 10-fold dilutions of PSC-RANTES (10 nM to 0.1 nM) for 2 h at 37°C in 5% CO₂ to monitor the effects of PSC-RANTES on CCR5 expression. Cells were then removed from the plates with 3 mM EDTA and washed with fluorescence-activated cell sorter (FACS) staining buffer (2% fetal bovine serum, 0.5% bovine serum albumin, and 0.02% sodium azide in PBS). U87.CD4.Hu-R5 cells were stained with 10 µl of R-phycoerythrin (PE)-conjugated mouse anti-human CCR5 monoclonal antibody 2D7 (BD Pharmingen) or 1 µl of a PE-conjugated isotype control (mouse immunoglobulin G2a, κ-1; BD Pharmingen). U87.CD4.Rh-R5 cells were stained with 10 µl of the PE-conjugated mouse anti-human monoclonal antibody 3A9 (BD Pharmingen), which cross-reacts with rhesus and cynomolgus macaque CCR5, or an isotype control. Cells were stained at room temperature for 30 min and then washed. Cells stained with 3A9 were fixed in 2% paraformaldehyde-PBS, while cells stained with 2D7 were analyzed live. Cells were analyzed using a FACScalibur flow cytometer with CellQuest software (BD Biosciences).

FACS was used to sort U87.CD4 cells that were transduced with the pBABE-RH-R5 plasmid on a Beckman Coulter Elite ESP (Case Comprehensive Cancer Center Flow Cytometry Core, Case Western Reserve University, Cleveland, OH). Cells were stained with the PE-conjugated mouse anti-Hu-CCR5 monoclonal antibody 3A9 as described above. CCR5-positive cells were single cell sorted into 2- to 96-well plates, and each cell was grown into populations. These cell populations were then tested for CCR5 expression using the 3A9 antibody, and a population of cells expressing high levels of CCR5 was chosen for subsequent experiments.

Calcium flux assays. U87.CD4.Hu-R5 or U87.CD4.Rh-R5 cells were washed and resuspended in a balanced salt solution (1.5 mM calcium chloride, 5 mM potassium chloride, 130 mM sodium chloride, 1.0 mM magnesium chloride, and 20 mM sodium HEPES [pH. 7.5]) and then incubated for 40 min with Fura-2AM dye (1 mg/ml in dimethyl sulfoxide) (Invitrogen). Cells were then spun down and resuspended in the balanced salt solution. A total of 1.5 ml of cells (3×10^6) was added to a cuvette and read on a fluorometer at 37°C. A 10 nM concentration of PSC-RANTES (kindly provided by Oliver Hartley) or RANTES (PreproTech) was added to the cells, and peak calcium efflux was measured using Allchroom Plus software. Ligand was added approximately 3 min after initiation of the fluorometer. Maximum calcium response was measured at approximately 6 min after initiation of the assay by adding 10 µl of 5-mg/ml digitonin. This was followed 2 min later by addition of 50 µl of 2 M Tris and 50 µl of 300 mM EGTA to measure the minimum calcium signal. Calcium peaks resulting from PSC-RANTES or RANTES binding to CCR5 were measured relative to the maximum and minimum calcium flux responses and compared between the two U87 cell types. Quantitation of calcium flux levels was dependent on the assumption that the cytosol of U87.CD4.CCR5 cells contains approximately 220 nM of baseline calcium.

Drug sensitivity assays. U87.CD4.Rh-R5 or U87.CD4.Hu-R5 cells were plated in 96-well plates (1.0×10^4 cells/well) 24 h before exposure to entry inhibitors. Cells were pretreated for 1 h with 10-fold dilutions of PSC-RANTES (100 nM to 0.0001 nM), TAK-779 (10 µM to 0.00001 µM), or enfuvirtide (100 µM to 0.0001 µM) and then infected in triplicate at a multiplicity of infection (MOI) of 0.01 infectious units (IU)/cell with the various chimeric viruses. At 24 h, cells were washed with PBS and medium was replaced on the cells containing the appropriate drug dilutions. Supernatants were collected every 2 days starting on day 3 to monitor for virus production using a radioactive RT assay as described above. Virus production as represented by RT activity was plotted against drug concentration and analyzed to assess the 50% inhibitory concentration (IC₅₀) of each virus to each entry inhibitor. RNA was extracted from virus collected at peak virus production during the drug sensitivity assay and was sequenced to ensure that no mutations in *env* arose during the time course of the drug sensitivity assay. Each assay was repeated in triplicate and in two independent experiments.

In vitro fitness assays. In vitro replicative fitness of chimeric viruses was measured using head-to-head competitions as previously described (6). U87.CD4.Rh-CCR5 or U87.CD4.Hu-CCR5 cells were plated in 48-well plates (1.5×10^4 cells/well) 24 h before infection. Head-to-head competitions were performed in quadruplicate between NL4-3/*env*_{m584} and NL4-3/*env*_{p3}. All competitions were performed at an equal and low MOI (0.0004 IU/ml) to maintain susceptible cells through several rounds of replication and to reduce the fre-

quency of recombination ($<0.01\%/1,000$ nucleotides) (4). Twenty four hours postinfection, the inoculum was washed off the cells with PBS and fresh medium was added back to the cells. Virus production was monitored by RT assay every 2 days, starting on day 3 postinfection. At peak virus production (day 7), cells were harvested from the infections and proviral DNA was extracted using the QiaAmp DNA blood kit (Qiagen). Oligonucleotide ligation assays were then performed to determine the levels of NL4-3/*env*_{m584} and NL4-3/*env*_{p3} by probing for K315 and N640 for the p3 wild type and R315 and D640 for the m584 mutant.

RESULTS

Evolution in SHIV_{SF162-p3} *env* genes derived from PSC-RANTES-treated rhesus macaques. In a previous study by Lederman et al., rhesus macaques vaginally pretreated with PSC-RANTES were protected from vaginal infection by the SHIV_{SF162-p3} virus at high doses of the drug (1 mM) (21). Plasma samples were obtained from multiple time points of infected rhesus macaques pretreated with these various doses (1 mM to 1 µM) of PSC-RANTES (Fig. 1a to f) or untreated. The envelope genes were PCR amplified and sequenced (8 to 25 different virus clones/sample) for possible amino acid changes that may be associated with drug resistance to PSC-RANTES (Fig. 1a to f). Analysis of the clonal *env* sequences revealed a relative homogeneity between the infected macaques and the inoculating virus SHIV_{SF162-p3} as well as minimal evolution during infection in untreated animals or those initially treated with vaginal administration of PSC-RANTES (see Fig. S1 in the supplemental material).

Of the few amino acid substitutions found in the SHIV_{SF162-p3} *env* genes, seven were unique to infected macaques in the presence of PSC-RANTES compared to the untreated controls (PBS only) and were consistently present in multiple samples (see Fig. S1 in the supplemental material). Only three *env* mutations were mapped to the gp120 coding region (V272I in bh49 with 10 µM PSC-RANTES treatment, F279V in m584 with 100 µM PSC-RANTES treatment, and K315R in m584 with 100 µM PSC-RANTES treatment). However, in animals bh49 and m584, the V272I and F279V mutations, respectively, were transient and only identified at day 49 and not day 21, 28, 43, or 77 postinfection (see Fig. S1 in the supplemental material). On the other hand, the K315R mutation was found throughout the m584 infection. This observation is in sharp contrast to the lack of any gp120 amino acid mutations in the remaining 34 (of 38) samples obtained from 13 macaques at multiple time points. It is important to note that sequence contamination was unlikely since unique non-synonymous nucleotide substitutions were identified in most samples.

The remaining four mutations in PSC-RANTES-treated macaques were found in the gp41 coding region. Two of these amino acid substitutions in gp41 were only identified at one time point during infection of different macaques. However, virus from the m584 macaque treated with 100 µM PSC-RANTES contained a mutation in the gp41 region of *env*. The N640D mutation was found in gp41 of m584 at all time points (days 21, 43, 49, and 77) (Fig. 1g and see Fig. S1 in the supplemental material). Only one other stable mutation was identified in PSC-RANTES-treated macaques. Animal bh49, treated with 10 µM PSC-RANTES, maintained a virus harboring the gp41 transmembrane mutation, V689I (Fig. 1g). Because this mutation was found in the transmembrane region of

FIG. 2. OLA to detect the frequency of K315R and N640D in SHIV-infected macaques. (a) Schematic representation of OLA. Details of this method are described in Materials and Methods. (b) Frequency of the K315R *env* mutation in different populations of virus isolated from the plasma of various macaques as indicated below each bar. The macaque animal number, time point postinfection, and PSC-RANTES doses are indicated. (c) Frequency of the N640D *env* mutation in different populations of virus isolated from the same macaques as indicated in panel b. Four independent assays were analyzed for each sample. Error bars represent a 10% deviation from the mean estimated as the error rate of this OLA based on standards run with the samples. The dotted line represents the limit of detection of this assay (0.4%).

gp41, it is less likely to impact drug susceptibility than mutations found in regions involved in the direct binding of receptors (gp120) or mutations in the extracellular gp41 domain, which may influence fusion kinetics. In addition, this V689I mutation was found in an animal treated with a low dose of PSC-RANTES, presenting a smaller selection pressure on the virus. Therefore, the likelihood of this mutation impacting drug sensitivity was minimal relative to the N640D gp41 mutation that we decided to pursue in this study. Future studies could address whether this or other transmembrane mutations impact PSC-RANTES sensitivity.

The K315R mutation in gp120 is found at the ³¹¹GPGX₃₁₅ crown of the V3 loop, a motif which may be involved in virus binding the N terminus of the CXCR4 or CCR5 coreceptors (11). The N640D mutation maps to the C-terminal helical region of gp41 and may mutate a putative N-linked glycosyla-

tion site. Mutations in the immediate vicinity of N640V such as N637K have been associated with altered rates of six- α -helix bundle formation and entry inhibitor drug resistance (26; M. J. Root, unpublished data). Any change in the entry kinetics of a virus could impact susceptibility to an HIV-1 entry inhibitor (28).

Estimating the frequency of K315R and N640D polymorphisms in the inoculating virus population. A modified, radio-labeled OLA (35) (Fig. 2a) was utilized to determine the prevalence of the K315R and N640D mutations in the (i) inoculating SHIV_{SF162-p3} population, (ii) virus populations from three macaque m584 samples (Fig. 2b and c, days 21, 43, and 49), (iii) virus populations from macaques (n400 and bh49) receiving low PSC-RANTES treatment (33 and 10 μ M, respectively), and (iv) virus populations infecting untreated macaques (al01, t619, and t314). This OLA analysis revealed that the

FIG. 1. Sequence analysis of SHIV_{SF162-p3} found in plasma of rhesus macaques pretreated with increasing doses of PSC-RANTES prior to SHIV_{SF162-p3} challenge. (a to f) Rhesus macaques pretreated with increasing concentrations of PSC-RANTES were monitored over time for plasma viral load as shown on each graph. Each line represents a different rhesus macaque treated with the dose of PSC-RANTES indicated at the top of each graph. RNA was extracted from plasma samples, and arrows indicate at what time points the *env* gene was successfully sequenced. The blue line in panel c shows m584 viral loads over time. (g) A map of the HIV-1 *env* gene showing the GPG crown of the V3 region of gp120 and the C-helical region of gp41. All animals pretreated with PSC-RANTES and carrying amino acid changes over multiple time points from the parental SHIV_{SF162-p3} are shown along with their mutations and time points. For complete sequences of these two regions of *env*, see Fig. S1 in the supplemental material.

N640D mutation in gp41 dominated in the m584-derived SHIV populations (>90%) but was below the limit of detection in the inoculating SHIV_{SF162-p3} virus and in the infected, untreated macaques (<0.4%) (Fig. 2c). The N640D mutation was detected at low percentage (0.77%) in the virus population derived from macaque bh49 treated with 10 μ M PSC-RANTES (Fig. 2c). Overall, the N640D mutation was enriched in the macaque m584 virus population by at least 75-fold over that in the inoculating virus population. Furthermore, this mutation was not selected in another five SHIV_{SF162-p3}-infected macaques.

Dominance of K315R mutation in the m584 populations was also evident by OLA, but in contrast to the N640D mutation, this V3 mutation could be detected at a 3% level in the inoculating virus population (Fig. 2b). Similar low percentages of K315R SHIV_{SF162-p3} mutants were found in the infected, untreated macaques and in macaques treated with low levels of PSC-RANTES. The untreated macaque t619 harbored K315R SHIV_{SF162-p3} at a 31% level. Regardless, there was at least a 25-fold enrichment of K315R virus in macaque m584 over the K315R virus in the inoculating virus or in the other SHIV_{SF162-p3}-infected macaques.

Sensitivity of NL4-3/env_{m584} and NL4-3/env_{p3} to entry inhibitors. We utilized a yeast-based recombination/cloning approach to generate chimeric viruses (outlined in Fig. S2 in the supplemental material) of NL4-3 containing the full-length *env* genes or gp120 coding regions from samples derived from macaque m584 (NL4-3/env_{m584} or NL4-3/gp120_{m584}) and from the inoculating stock of SHIV_{SF162-p3} (NL4-3/env_{p3} or NL4-3/gp120_{p3}). Given that infection in the presence of PSC-RANTES occurred in a rhesus macaque model, it seems most relevant to study the impact of the *env* mutations in the context of Rh-CCR5 (designated Rh-R5 in the cell lines discussed below). Thus, stable U87.CD4.Rh-R5 cells were created as described in Materials and Methods using a pBABE-Rh-CCR5 vector. U87.CD4.Rh-R5 or U87.CD4.Hu-R5 cells had comparable levels of Rh- and Hu-CCR5 surface expression (data not shown). PSC-RANTES (1, 10, and 100 nM) effectively down-regulates Hu-CCR5 (27; data not shown) as well as the Rh-CCR5 (Fig. 3a) from the surface of the U87 cells as shown by flow cytometry. Furthermore, both the native RANTES and the analog, PSC-RANTES, induced calcium influx through these Rh- and Hu-CCR5 G-coupled protein receptors (Fig. 3b). Finally, R5 HIV-1 could enter and efficiently replicate in the U87.CD4 cells expressing Rh-CCR5 or Hu-CCR5 as shown in the no-drug controls of the drug sensitivity study (Fig. 4b and c).

The sensitivities of NL4-3/env_{m584}, NL4-3/env_{p3}, and the laboratory strain HIV-1_{ADA} to various entry inhibitors were compared in both U87.CD4.Rh-R5 and U87.CD4.Hu-R5 cells. The NL4-3/env_{m584} was 7-fold more resistant to PSC-RANTES than NL4-3/env_{p3} in U87.CD4.Rh-R5 cells ($P < 0.05$, two-tailed *t* test) and only 5.5-fold more resistant in U87.CD4.Hu-R5 cells ($P < 0.1$) (Fig. 4a). Of greater significance is the incomplete inhibition (~75%) of NL4-3/env_{m584} by PSC-RANTES at even a 100 nM concentration (23-fold over the IC₅₀), whereas the same drug concentration inhibited >99% of NL4-3/env_{p3} replication in U87.CD4.Rh-R5 (Fig. 4b). The lack of complete inhibition of NL4-3/env_{m584} replication by PSC-RANTES on U87.CD4.Rh-R5 cells is best exem-

plified through the direct visualization of the radiolabeled products from the virus-derived RT activity, used to quantify the drug sensitivity assays (Fig. 4b). In the U87 cells expressing Hu-CCR5, the slight shift in IC₅₀ values was accompanied by complete inhibition by 10 and 100 nM PSC-RANTES (Fig. 4c).

Cloning of the gp120 coding region into NL4-3 resulted in an NL4-3/gp120_{m584} virus (K315R) that was twofold more resistant to PSC-RANTES compared to the NL4-3/gp120_{p3} virus in U87.CD4.Rh-R5 cells ($P < 0.05$) (Fig. 4a). Again, 100 nM PSC-RANTES was unable to completely inhibit NL4-3/gp120_{m584} (K315R) virus compared to 100% inhibition of the NL4-3/gp120_{p3} virus (data not shown). In the U87.CD4.Hu-R5 cells, there was an 11-fold difference in sensitivity of NL4-3/gp120_{p3} and NL4-3/gp120_{m584} to PSC-RANTES inhibition ($P < 0.01$) (Fig. 4a) with complete inhibition by 100 nM of the drug (data not shown). These findings suggest that K315R in NL4-3/gp120_{m584} plays a significant role in PSC-RANTES resistance with Rh-CCR5. However, N640D, as part of the m584 gp41 domain, might stabilize the m584 gp120 coding region, resulting in a further increase in PSC-RANTES resistance.

Decreased sensitivity (or resistance) to PSC-RANTES with m584 versus the SHIV_{SF162-p3} viruses (Env or gp120 chimeric viruses) was reflected in a similar decrease in sensitivity to TAK-779 (Fig. 4a). As with PSC-RANTES, this resistance was less pronounced in the U87 cells expressing Hu-CCR5 than in those expressing Rh-CCR5 because TAK-779 was unable to completely block m584 virus replication (as both the Env or gp120 NL4-3 chimeric virus) in the U87.CD4.Rh-R5 cells (data not shown). When compared to NL4-3/env_{p3}, NL4-3/env_{m584} showed a trend toward hypersusceptibility to the fusion inhibitor enfuvirtide in U87.CD4.Hu-R5 cells (Fig. 4a). In this case, NL4-3/env_{m584} harbors the N640D substitution in the C-HR, which could alter the kinetics of six- α -helix bundle formation. Sensitivities of NL4-3/gp120_{m584} and NL4-3/gp120_{p3} to enfuvirtide on U87.CD4.Hu-R5 were not significantly different; however, NL4-3/gp120_{m584} was significantly more resistant to enfuvirtide on U87.CD4.Rh-R5 cells.

In vitro competition assays to determine the replicative fitness of PSC-RANTES-resistant m584. Similar levels of NL4-3/env_{m584} and NL4-3/env_{p3} virus were observed at 7 days postinfection in both U87.CD4.Rh-R5 and U87.CD4.Hu-R5 cells (Fig. 5a). Fitness differences are difficult to discern during mono-infections, due to variability between cultures in mono-infections, the lack of internal controls (i.e., competitor virus), and the logarithmic growth rate of virus production (6, 34). To obtain a more refined measure of relative fitness, quadruplicate head-to-head competitions were performed between NL4-3/env_{m584} and NL4-3/env_{p3} using both U87.CD4.Rh-R5 and U87.CD4.Hu-R5 cell lines (Fig. 5b). Given that these viruses differ by only two amino acids, OLAs instead of our more standard heteroduplex tracking assays (HTAs) were used to analyze dual virus production. Oligonucleotide sets were used to probe and quantify for both the K and R at position 315 (NL4-3/env_{p3} and NL4-3/env_{m584}, respectively) and for the N and D at position 640 (NL4-3/env_{p3} and NL4-3/env_{m584}, respectively). In competitions performed in U87.CD4.Rh-R5 cultures, the PSC-resistant variant, NL4-3/env_{m584}, was dramatically more fit

FIG. 3. Calcium flux assays and flow cytometry to verify the functional expression of Rh-CCR5 in U87.CD4.Rh-R5 cells compared to Hu-CCR5 in U87.CD4.Hu-R5 cells. (a) Change (fold) in CCR5 surface expression relative to the isotype control was measured following treatment with different levels of PSC-RANTES (0.1 to 100 nM). Bars represent mean fluorescence intensity (MFI) associated with surface staining for Rh-CCR5 with the monoclonal antibody 3A9. Error bars in panels a and b represent the standard deviation of triplicate independent assays performed for each condition. (b) Calcium flux response was measured following treatment with RANTES (10 nM) and PSC-RANTES (10 nM) in U87.CD4.Hu-R5 or U87.CD4.Rh-R5 cells loaded with Fura-2AM dye. The bar graph indicates the cytosolic change in calcium upon addition of each ligand. Examples of the calcium response peaks measured on a fluorometer in each cell line are shown.

than the inoculating virus, NL4-3/env_{p3} (Fig. 5d) ($P < 0.001$). This fitness difference was dampened in competitions performed in the U87.CD4.Hu-R5 cultures (Fig. 5d). As discussed below, the increased replicative fitness of NL4-3/env_{m584} over NL4-3/env_{p3} reflects the increased resistance of this mutant to PSC-RANTES inhibition. This result is also consistent with previous results showing that increased fitness and host cell entry efficiency can be associated with decreased sensitivity to PSC-RANTES, AD101, and other entry inhibitors (2, 25).

DISCUSSION

Inexpensive, well-tolerated, and inhibitory antiviral compounds are all required attributes of an HIV-1 microbicide to prevent sexual transmission. However, as clearly demonstrated by the failure of nonoxynol-9, cellulose sulfate, and Carraguard to prevent HIV-1 transmission (36), success of an HIV-1 microbicide is not solely dependent on these general attributes (17, 18). Recently, the focus for HIV-1 microbicides has shifted to the use of specific antiretroviral or anti-HIV-1 compounds in either vaginal/rectal administrations or as a daily oral prophylactic. Several drugs inhibiting different steps in the HIV-1 life cycle have been proposed, but HIV-1 entry inhibitors have gained the most attention for vaginal microbicide use. HIV-1 entry inhibitors such as microbicides are advantageous because these drugs (i) prevent the entry or the initiation of retroviral life cycle, (ii) do not require uptake by the host cell, and (iii) can target viral receptors (in some cases) rather than directly target the virus.

Preliminary studies with PSC-RANTES, BMS-378806, CMPD167, and C52L, have all shown effective prevention of macaque infection by an R5 SHIV in the vaginal treatment/challenge model (21, 37). The caveats to this success are also apparent. First, large amounts of these entry inhibitors were required to block macaque infection. In the case of PSC-RANTES, the 1 mM concentrations required for full protection exceeded the R5 HIV-1 inhibitory concentrations (IC_{50} of 1 to 10 nM) in tissue culture by at least 10,000-fold (even with macaque peripheral blood mononuclear cells). Second, the prevention of macaque infection has only been observed with a single R5 SHIV, an SF162 strain that was adapted to macaque infection (12) and then passaged twice (p2) or three times (p3) in tissue culture. Incidentally, this strain is exquisitely sensitive to many entry inhibitors, including PSC-RANTES. Third, and only pertaining to PSC-RANTES, the role of CCR5 receptor downregulation as the “favored” inhibitory mechanism for a PSC-RANTES microbicide can be questioned with the selection of resistant SHIV variants. The ability of the drug to bind CCR5 and act as a competitive inhibitor for HIV-1 entry appears to be the dominant mechanism of inhibition with prolonged incubations with PSC-RANTES (22). Finally, the homogeneity of SHIV_{SF162-p3} does not necessarily provide an appropriate model for human sexual transmission where the inoculating virus from the donor is not homogeneous. As a consequence of these limitations in the macaque model, the barrier for selecting resistance to an anti-HIV microbicide (such as PSC-RANTES) has been set very high and possibly beyond physiological relevance.

In this study, we report the first identification and characterization of viral resistance to a vaginal microbicide. Resistance emerged to the entry inhibitor PSC-RANTES in a macaque vaginally treated with the drug and then exposed to SHIV_{SF162-p3}. To identify possible resistance mutations, we first examined the SHIV_{SF162-p3} env sequences of the macaques infected in the presence of PSC-RANTES or with the vehicle control using the vaginal treatment/challenge model. At the lower concentrations of PSC-RANTES, the virus that established and maintained infections was nearly identical to the SHIV_{SF162-p3} inoculating virus. In addition, any emerging or selected mutations were either transient, highly conserva-

FIG. 4. Drug sensitivity of chimeric SHIV_{SF162-p3} viruses to PSC-RANTES, TAK-779, and enfuvirtide. (a) IC₅₀s of NL4-3/env_{m584}, NL4-3/env_{p3}, NL4-3/gp120_{m584}, NL4-3/gp120_{p3}, or HIV-1_{ADA} to PSC-RANTES, TAK-779, and enfuvirtide (ENF) on either U87.CD4.Rh-R5 cells (left) or U87.CD4.Hu-R5 cells (right). Error bars depict the standard deviation of independent assays performed in at least three replicates. (b and c) Radioactive [α -³²P]TTP RT assay X-ray film images representing the amount of NL4-3/env virus production in the presence of different concentrations of PSC-RANTES or a no-drug control on either U87.CD4.Rh-R5 or U87.CD4.Hu-R5 cells, respectively. The more intense the black spot, the more virus is produced in the presence of each concentration of drug by day 7 postinfection (PI). *P* values are derived from one-tailed Mann-Whitney or Student's *t* tests. Only *P* values of <0.05 for the NL4-3/env or gp120_{m584} viruses relative to the NL4-3/env or gp120_{p3} viruses, respectively, are presented in each panel.

tive, or were observed in the macaques vaginally infected with SHIV_{SF162-p3} in the absence of PSC-RANTES. However, two specific mutations, K315R in gp120 and N640D in gp41, were selected within 21 days in one of the macaques (m584) infected in the presence of a higher PSC-RANTES concentration (100

μM). The dominance of the K315R and N640D mutations in macaque m584 treated with 100 μM PSC-RANTES represents at least a 25-fold and 75-fold enrichment/selection (respectively) over that present in the inoculum. These mutations were stable throughout macaque m584 infection, but unfortunately, there was no detectable virus in the plasma at day 15 postinfection to determine if the macaque was infected by the K315R/N640D SHIV_{SF162-p3} or if these mutations emerged during infection. The former hypothesis of immediate selection/infection is favored because m584 and all macaques in this study were treated with a vaginally administered dose of PSC-RANTES at 15 min prior to challenge. Given a single administration of PSC-RANTES, it was unlikely that macaque m584 was infected with a “wild-type” SHIV_{SF162-p3} for an extended period (up to 21 days) prior to the emergence and dominance of the PSC-RANTES-resistant strain.

The NL4-3/env_{m584} strain showed a modest sevenfold resistance to NL4-3/env_{p3}, but more importantly, PSC-RANTES failed to completely inhibit NL4-3/env_{m584} even at 100 nM, i.e., a concentration that completely blocks replication of NL4-3/env_{p3} and all other R5 HIV-1 isolates tested (data not shown). This PSC-RANTES resistance was most demonstrable in cell lines expressing Rh-CCR5 (Rh-R5) as opposed to Hu-CCR5 (Hu-R5). These findings suggest a selection of a SHIV_{SF162-p3} clone more adapted to use of Rh-CCR5 in the context of PSC-RANTES. This observation is further supported by increased replicative fitness of the NL4-3/env_{m584} in the U87.CD4.Rh-R5 as compared to fitness in U87.CD4.Hu-R5. Mutations K315R and N640D were likely selected or co-evolved in SHIV_{SF162-p3} to maintain fitness in the context of Rh-CCR5 while avoiding drug inhibition. Previous studies have shown that resistance to PSC-RANTES or AD101 is often linked to an increase in HIV-1 entry efficiency and replicative fitness (2, 25).

The K315R mutation is located one amino acid downstream from the highly conserved GPG motif in the crown of the V3 loop and is part of the β-turn located in this region of gp120 (13). This region is associated with the direct interaction of CD4-bound gp120 with the second extracellular loop of CCR5 (14). It must be noted that the majority of HIV-1 laboratory strains (HIV-1_{JRFL}, HXB2, HIV-1_{ADA}, and HIV-1_{BAL}) and primary isolates contain arginine at gp120 position 315 and that lysine at this position is rarely tolerated despite charge and side chain size conservation (9, 11). In SHIV_{SF162-p3}, a lysine at position 315 was selected over arginine during passages through macaques and adaptation for vaginal infection (12). The gp41 N640D mutation, on the other hand, was not present in the inoculating SHIV_{SF162-p3}, in the original HIV-1_{SF162}, or in any other SHIV-infected macaque. Nonetheless, both asparagine and aspartic acid are the most frequently found amino acids found at gp160 position 640 in primary subtype B HIV-1 isolates (Los Alamos database Epilign). This asparagine may or may not be an N-linked glycosylation site in the C-heptad repeat of gp41 but is found on the outer face of the alpha-helix following the formation of the six-alpha-helix bundle. The presence of this mutation in the full-length NL4-3/env_{m584} sequence rendered the virus more sensitive to enfuvirtide than the NL4-3/gp120_{m584} virus counterpart lacking this mutation but only on the rhesus cell line. This suggests a role for this mutation to alter entry capacity, to increase PSC-

FIG. 5. Fitness of SHIV_{SF162-p3} env chimeric viruses. (a) Virus production during monoinfections of either NL4-3/env_{m584} or NL4-3/env_{p3} viruses on either U87.CD4.Rh-R5 or U87.CD4.Hu-R5 cells. Virus production was measured by HTA analysis of the proviral DNA found in cells at the time point of peak virus production. Radiolabeled probes were used during HTA, and the intensity of the band on the gel represents the amount of proviral DNA bound by the probe measured by a phosphorimager. Error bars represent the standard deviation of quadruplicate independent experiments for each condition. PI, postinfection. (b) Schematic representation of an in vitro fitness assay. U87.CD4.Rh-R5 or U87.CD4.Hu-R5 cells were infected with both NL4-3/env_{m584} and NL4-3/env_{p3} at equal MOIs of 0.0004. At peak virus production, cells were harvested, proviral DNA was amplified in either the *vif* gene or *env* gene, and DNA levels were measured by OLA. (c) Mean relative fitness values for head-to-head competitions between the NL4-3/env_{p3} and NL4-3/env_{m584} chimeric viruses. ***, $P < 0.001$.

RANTES resistance and, inadvertently, to increase sensitivity to enfuvirtide, specifically in the rhesus cell line. An adjacent mutation, N637K, can disrupt an N-linked glycosylation site and confer 6.8-fold resistance to C34, a fusion inhibitor (26). Other amino acid substitutions at position 637 also alter sensitivity to both enfuvirtide and the fusion inhibitor 5-helix (5). Preliminary results suggest increased rates of cell fusion by these 637 mutations may play a role in resistance to fusion inhibitors (M. J. Root, unpublished data).

In conclusion, this PSC-RANTES-resistant variant in rhesus macaques is the first evidence for selection/infection by HIV or SHIV resistant to a vaginally applied microbicide. It is important to note that the selection of PSC-RANTES-resistant virus was only identified in 1 of 25 macaques vaginally treated with the drug. The random selection of a variant with higher fitness in the rhesus macaque cells, rather than selection for drug resistance, cannot be entirely ruled out. However, we have previously reported a direct correlation between replicative fitness, increased efficiency of host cell entry, and decreased sensitivity to PSC-RANTES using a set of HIV-1 clones harboring natural polymorphisms in the V3 loop (22). Increased fitness may lead to decreased susceptibility to entry inhibitors (2, 22), but selected resistance to several CCR5 antagonists and fusion inhibitors can also result in decreased replicative fitness (in the absence of drug) (2, 23). Nonetheless, any resistance to PSC-RANTES was unexpected considering (i) the single vaginal rather than systemic drug application, (ii) the putative mechanism of PSC-RANTES inhibition, (iii) the exquisite PSC-RANTES sensitivity of this SHIV compared to primary HIV-1 isolates, and (iv) the homogeneity of the inoculating virus. In heterosexual transmission, the diversity of inoculating virus from the donor is almost never clonal, even if the donor is in an acute stage of disease. Given the current high barrier for HIV-1 resistance and problems that could ensue from this microbicide model, the production of new R5-tropic SHIV variants that could be combined in macaque challenges would aid in the proper testing and identification of suitable microbicide candidates for future human use to prevent HIV-1 transmission. It is also important to consider that at the highest dose, PSC-RANTES was fully protective and

that this resistant mutant emerged at a slightly lower dose. Due to the nature of microbicides, it is highly conceivable that after application, there will be a window of time when the drug is potent followed by a period when suboptimal doses of the drugs may provide the necessary conditions for emergence of drug resistance.

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