Reversion of the M184V Mutation in Simian Immunodeficiency Virus Reverse Transcriptase Is Selected by Tenofovir, Even in the Presence of Lamivudine

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The methionine-to-valine mutation in codon 184 (M184V) in reverse transcriptase (RT) of human immunodeficiency virus type 1 (HIV-1) or simian immunodeficiency virus (SIV) confers resistance to (–)-2′-deoxy-3′-thiacytidine (3TC; lamivudine) and increased sensitivity to 9-[2-(phosphonomethoxy)propyl]adenine (PMPA; tenofovir). We have used the SIV model to evaluate the effect of the M184V mutation on the emergence of resistance to the combination of 3TC plus PMPA. A site-directed mutant of SIVmac239 containing M184V (SIVmac239-184V) was used to select for resistance to both 3TC and PMPA by serial passage in the presence of increasing concentrations of both drugs. Under these selection conditions, the M184V mutation reverted in the majority of the selections. Variants resistant to both drugs were found to have the lysine-to-arginine mutation at codon 65 (K65R), which has previously been associated with resistance to PMPA in both SIV and HIV. Similarly, in rhesus macaques infected with SIVmac239-184V for 46 weeks and treated daily with (–)-2′-deoxy-5-fluoro-3′-thiacytidine [(–)-FTC], there was no reversion of M184V, but this mutation reverted to 184 M in all three animals within 24 weeks of treatment with (–)-FTC and PMPA. Although the addition of PMPA to the (–)-FTC therapy induced a decrease in virus loads in plasma, these loads eventually returned to pre-PMPA levels in each case. All animals receiving this combination developed the K65R mutation. These results demonstrate that the combination of PMPA with 3TC or (–)-FTC selects for the K65R mutation and against the M184V mutation in SIV RT.

The rapid emergence of drug-resistant mutants of human immunodeficiency virus (HIV) has proven to be a major obstacle in antiviral therapy for AIDS (7, 32, 36, 63). Even highly active antiretroviral therapy has been limited by the emergence of multidrug-resistant HIV (32, 46, 61). This has led to efforts to identify drug combinations in which resistance to one drug results in a mutant virus that suppresses normal resistance to a second drug or in a mutant virus that is hypersensitive to other drugs (2, 27, 28, 35, 52).

One mutation that results in phenotypic changes in HIV type 1 (HIV-1) that are useful in certain drug combinations is the methionine-to-valine mutation in codon 184 (M184V) of reverse transcriptase (RT). This mutation arises rapidly in therapy with the oxathiolane nucleosides, (–)-2′-deoxy-3′-thiacytidine (3TC; lamivudine) and (–)-2′-deoxy-5-fluoro-3′-thiacytidine [(–)-FTC], and results in high-level (>100-fold) resistance to these drugs (49, 55). This is often preceded by emergence of a methionine-to-isoleucine mutation in codon 184 (M184I), which is quickly replaced by M184V (22, 50). This M184V mutation is located in the highly conserved YMDD motif of RT, which is directly involved in binding the incoming nucleotide during reverse transcription (19, 20, 24, 53). It results in decreased processivity (1, 3, 21, 23, 51) and increased fidelity (11, 41, 62) of the DNA polymerase activity

of RT in biochemical assays. However, the increase in fidelity was less than twofold in an M13 phage-based assay that scored the overall mutation rate after transfection of RT products into bacteria (9). M184V mutants of HIV-1 also have reduced replication rates in certain cell lines (1, 35, 51), and they have a broad array of changes in susceptibility to nucleoside analogs (15, 28, 35, 54, 55, 64).

A major effect of the M184V mutation is to suppress phenotypic resistance to 3′-azido-3′-deoxythymidine (AZT; zidovudine) when M184V is present along with mutations that normally confer resistance to AZT (28, 55). Clinical results show that the 3TC-plus-AZT combination produces a much more sustained response than would be expected based on results with these drugs individually (10, 28). Resistance to this combination requires more complex patterns of mutations than is required when either drug is used in monotherapy (39). An important aspect of 3TC-plus-AZT combination therapy is the frequent development of the M184V mutation despite the suppressive effect that it has on common AZT resistance mutations (26, 37, 39, 42). In contrast, another mutation that causes phenotypic suppression of AZT resistance, Y181C (27), emerges with nevirapine monotherapy (45, 47) but not in the presence of the combination of AZT plus nevirapine (47). Resistance to this combination requires alternate mutations for nevirapine resistance (47). In the case of 3TC plus AZT, alternative mutations that give resistance to 3TC are less common.

The M184V mutation in HIV-1 RT has also been shown to

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make HIV-1 two- to fivefold more sensitive to the acyclic nucleotide analog 9-[2-(phosphonomethoxy)propyl]adenine (PMPA; tenofovir) (35, 64). Although it has been suggested that this may make a combination of 3TC and PMPA more efficacious (35), this has not been demonstrated conclusively (34). In the work presented here, we have studied resistance to the combination of 3TC plus PMPA using simian immunodeficiency virus (SIV) as a model system. SIV is similar to HIV-1 in susceptibility to 3TC (4) and to PMPA (57). In addition, as in HIV-1, M184V and K65R mutations in SIV RT confer resistance to 3TC and PMPA, respectively (4, 57, 58). We report here the effects of selection with 3TC plus PMPA upon the drug-susceptibilities of SIV.

MATERIALS AND METHODS

Viruses and cells. Virus produced from two SIVmac239 molecular half-clones was used as wild-type SIV for these studies. The SIVmac239 half-clones (containing a full-length open reading frame for the *nef* gene) were kindly provided by Paul Luciw, University of California, Davis (33). Infectious virus was obtained by transfecting CEMx174 cells (48) with two SIVmac239 half-clones using electroporation. The 5' half-clone of SIVmac239 containing the M184V mutation in RT was kindly provided by Mark Wainberg, McGill University, Montreal, Quebec, Canada (4). PMPA-resistant strains of SIVmac251 were isolated from rhesus macaques treated with PMPA monotherapy as previously described (57, 60). The SIVmac055 isolate contained the K65R, N69T, R82K, A158S, and S211N mutations in RT. The SIVmac385 isolate contained the K65R, N69S, and I118V mutations in RT. Levels of PMPA resistance for these two variants were no different from those for isolates that had only the K65R mutation. SIV isolates were grown and maintained in CEMx174 cells using RPMI 1640 medium (GIBCO, Invitrogen, Carlsbad, Calif.) supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-Products, Woodland, Calif.) that had been heat inactivated for 30 min at 56°C, 100 U of penicillin per ml, 100 µg of streptomycin per ml, and 2 mM L-glutamine (GIBCO). Stocks of SIV mutants were grown in the presence of drug at a concentration equivalent to that in the passage of selection from which that mutant was collected. When virus was detected in cultures by p27 antigen capture enzyme-linked immunosorbent assay (ELISA) or focal infectivity assay (FIA) (see below), medium containing drug was removed and cells were incubated for 2 days in growth medium without drug. Virus stocks were prepared from the supernatants of these cultures by centrifugation at 600 × g for 10 min and were stored at -70°C.

The p27 antigen capture ELISA was performed as described previously (31), except that the coating of plates was modified. Maxisorp Nunc-Immuno plates (Fisher Scientific) were coated with streptavidin (Southern Biotechnology Associates, Inc., Birmingham, Ala.) at a concentration of 2 µg/well in a volume of 200 µl of coating buffer (0.1 M sodium carbonate, pH 9.6) per well. The plate contents were incubated at room temperature overnight. After being washed, the biotinylated antigen capturing monoclonal antibody, 55-2F12, was then added to the plates, and the rest of the assay was performed as previously described (31).

HI-JC.37 cells (43) were used for the FIA. These cells are the JC.37 clones of HeLa HI-J (43), which naturally express CXCR4, and have been modified to stably express both CD4 and CCR5. They are permissive for all HIV isolates that have been tested, including both macrophage- and T-cell-tropic virus isolates (43). In addition, HI-JC.37 are permissive for SIV infection (25). These cells were maintained in Dulbecco modified Eagle medium, supplemented with FBS, penicillin, streptomycin, and L-glutamine at concentrations described above. All cultures were maintained at 37°C with a humidified 5% CO₂ atmosphere.

Chemicals and drugs. PMPA was provided by Gilead Sciences, Inc., (Foster City, Calif.); 3TC and (-)-FTC were provided by Raymond Schinazi, Emory University, Decatur, Ga. All other chemicals were reagent grade or better.

FIA. Inhibition of SIV infection by antiviral drugs was quantified by an FIA, which was similar to those described previously for HIV-1 and feline immunodeficiency virus (5, 44). HI-JC.37 cells were seeded into 96-well microtiter plates at a density of 5.0×10^3 cells per well and were incubated overnight at 37°C. Medium was then removed and replaced with 100 µl per well of growth medium or growth medium containing a drug. The plate contents were incubated for 1 h at 37°C to allow the cells to convert the drug to its active form. These cells were then incubated for 1 h at 37°C with 100 µl of Dulbecco modified Eagle medium per well plus 0.1% FBS containing 20 to 60 focus-forming units of wild-type or mutant SIV and the appropriate drug concentration. After 1 h, 100 µl of growth medium with drug and FBS was added to bring the final concentration to 10%

TABLE 1. Primers used in this study

Primer name	3' base	Primer sequence (5'-3')
SIV-RT1	2788	GGATTAAAGGGACAATCATGACAGGG
SIV-RT8 (R)	3385	CCATATACTGGACTAAGGTC
SIV-RT10 (R)	3987	GGGACCTGTCCCCAGATCACTA
SIV-RT16 (R)	3635	TTGAGCTGCCCAATTTAATACTCC
239-BamHI	1860	CAGGAGATGGATCCAACCTGG
239-2459	2459	ACCCAGCTGTGGATCTG
239-2565 (R)	2565	TACTGGTCTCTCCAAGAG
239-K65R (R)	3029	GTTCCTTATCCTTTCTTTATAGCAAATG
239-K65R (F)	3057	CATTGTCTATAAAGAGAAAGGATAAGAAC
239-3461 (R)	3461	CTATTCAAGAGTTCCTTTGACTG

FBS while maintaining the desired drug concentration. In some cases, virus was used from selections that contained drug. In these cases, medium was removed after 1 h of viral adsorption and was replaced with growth medium containing 10% FBS and the appropriate drug concentration. Cells were incubated for 4 days at 37°C and a humidified 5% CO₂ atmosphere. Cells were fixed for 5 min with methanol and were washed twice with TNE (0.01 M Tris-HCl, pH 7.5, containing 0.15 M NaCl and 0.002 M EDTA). Cells were then washed once with TNE plus 1% FBS. Immunostaining was performed by incubating the cells for 30 min with 100 µl of a 1/2,500 dilution of polyclonal antiserum per well, which was obtained from rhesus macaques that had been infected with SIV. Cells were washed twice with TNE plus 1% FBS and were then incubated for 40 min with 100 µl of a 1/1,000 dilution of horseradish peroxidase-conjugated rabbit anti-monkey immunoglobulin (Sigma-Aldrich, St. Louis, Mo.). Cells were washed twice with TNE plus 1% FBS, and foci of infected cells were visualized by reacting the antibody-bound monolayers for 30 min, in the dark, with a solution of amino-ethylcarbazole at 0.2 mg/ml and with H₂O₂ (1 µl of 30% H₂O₂ per 2 ml) in 0.05 M sodium acetate buffer, pH 5. Foci appeared as red cells against an unstained background and were counted under a dissecting microscope at 30 to 100 × magnification.

Data were plotted as a percentage of control plaques (no drug) versus inhibitor concentration. Concentrations required to inhibit focus formation by 50% (EC₅₀s) were obtained directly from the linear portion of these plots by using a computer-generated regression line. Within an experiment, each value represents the mean of four replicate wells. Results from three or more independent experiments were used to derive the EC₅₀ plus or minus standard error.

Selection of drug-resistant mutants. SIV mutants resistant to PMPA, 3TC, or 3TC plus PMPA were obtained by passage of SIVmac239 or of indicated mutants in CEMx174 cells in medium containing drugs at concentrations that were increased in a stepwise fashion with each passage. Each selection was carried out in triplicate. For example, in the first passage of selection with PMPA, approximately 10⁶ CEMx174 cells were incubated in each of three 25-cm² flasks for at least 1 h in RPMI 1640 with 0.1% FBS and 5 µM PMPA. These cells were then infected with a cell-free supernatant containing approximately 1,000 focus-forming units of SIV grown from the molecular clone SIVmac239. After one additional hour, FBS was added to each selection to bring the total amount of FBS to 10%. Medium and drug were replaced every 2 or 3 days, and cells were removed and subcultured as necessary. Cultures were monitored by p27 ELISA as described above every 2 or 3 days. When virus production was apparent, cell-free supernatants from each of the first-passage cultures were used to infect flasks of CEMx174 cells for the second passage of selection in the presence of 10 µM PMPA. The drug concentration for each subsequent passage of selection increased twofold. In 3TC selections, 1 µM 3TC was used in the first passage, and in selections with PMPA and 3TC, 5 and 1 µM were used, respectively.

In the work reported here, two sets of selection conditions were used. In the first set of conditions, cultures were monitored by p27 ELISA and considered positive when culture samples yielded 5 ng or more of SIV p27 antigen per ml. Each new passage was started by infecting 10⁶ CEMx174 cells with 100 µl of cell-free supernatant from the previous passage. In the second set of conditions, p27 ELISA and FIA were used to monitor cultures; these were considered positive when infectious virus was detected by FIA. In the second experiment, 1,000 focus-forming units were used to infect 10⁶ CEMx174 cells to begin each new passage. In later passages, if 1,000 focus-forming units did not establish infection, then 10,000 focus-forming units were used to infect each new culture.

Nucleic acid preparation and sequence analysis. Total cellular DNA containing proviral DNA was extracted from infected CEMx174 cells and amplified by using nested PCR based on methods previously described (58). For samples in which the entire RT region of proviral DNA was sequenced, the methods pre-

TABLE 2. Genotypes of the first 195 codons of RT from selections with 3TC plus PMPA^a

Virus and selection	Genotype found after passage no.:						
	1	2	3	4	5	6	7
SIVmac239 (WT)^d							
[3TC]/[PMPA] (μM)	1/5	2/10	4/20	8/40	16/80	32/160	64/320
Selection 1	+	+	+	—			
Selection 2	+	+	+	K66K/R	K65R	—	
Selection 3	+	+	+	—			
Selection 4	+	+	+	K65R	K65R ^b	—	
Selection 5	+	+	+	K65R	K65R ^b	—	
Selection 6	+	+	+	—			
SIVmac239-184V							
[3TC]/[PMPA] (μM)	1/5	2/10	4/20	8/40	16/80	32/160	64/320
Selection 1	M184V	+	—				
Selection 2	WT	WT	WT	K65R	K65R	—	
Selection 3	M184V	WT	WT	K65R	K65R	—	
Selection 4	M184V	M184V	K65R M184V	K65R R82K/R M184V	K65R R82K/R I118I/V M184V ^b	K65R R82R/K I118V M184V V465V/I ^c	—
Selection 5	M184V	M184V	I145G M164I M184V ^b	—			
Selection 6	M184V	WT	K65R	K65R	K65R N69S V181I ^b	K65R N69S I118V V181I ^c	—
SIVmac239-184V							
[3TC]/[PMPA] (μM)	100/5	100/10	100/20	100/40	100/80	100/160	100/320
Selection 1	+	+	K65R M184V	—			
Selection 2	+	+	K65R M184V	K65R M184V	K65R R82K M184V	—	
Selection 3	+	+	K65R M184V	—			
Selection 4	+	+	—				
Selection 5	+	+	K70T M184V ^b	—			
Selection 6	+	+	M164I M184V	—			

^a The detection of virus replication is designated by a + or by the genotype that was determined by sequencing. Selections 1 to 3 were carried out under the first set of conditions, and selections 4 to 6 were carried out under the second set of conditions as described above (Materials and Methods). —, no virus replication was detected by p27 ELISA.

^b A titer of 10,000 focus-forming units was used to start the selection, as opposed to the 1,000 focus-forming units otherwise used.

^c The complete RT region of the *pol* gene was sequenced.

^d WT, wild type.

viously described were followed for the nested PCR and sequencing reactions (58). For all other samples, we sequenced only the first 195 codons, using primer RT10 (R) (Table 1) in the second round of nested PCR instead of SIV-4615 (R). Primers 239-2786 and SIV-RT3 were used to sequence amplified DNA as previously described (58).

For nested RT-PCR, RNA was extracted from 100 μl of plasma by using a silica-guanidine-thiocyanate extraction protocol (6) and was eluted with 50 μl of RNase-free water. Synthesis of cDNA and the first round of PCR were carried out using the Invitrogen One-Step RT-PCR mix (Invitrogen, Carlsbad, Calif.) according to the manufacturer's recommended conditions with 10 μl of RNA and a 2 μM concentration of each of the primers 239-2675 (58) and RT16 (R) (Table 1). Reaction conditions were 50°C for 30 min and 94°C for 2 min, followed by 45 cycles of 94°C for 15 s, 50°C for 30 s, and 72°C for 90 s, and finishing with a 5-min extension at 72°C. A second-round PCR was carried out using Gene Choice RedPOL *Taq* polymerase (PGC Scientifics, Gaithersburg, Md.) under the manufacturer's recommended conditions with 1.5 mM MgCl₂, 2 μl of first-round RT-PCR mixture, primers SIV-RT1 and 239-3461 (R) (Table 1), and distilled water in 100 μl of total volume. Reaction conditions were 94°C for 60 s,

followed by 30 cycles of 94°C for 15 s, 53°C for 30 s, and 72°C for 45 s, and finishing with a 5-min extension at 72°C.

Site-directed mutagenesis. SIVmac239 containing the K65R mutation in RT was made by site-directed mutagenesis by using PCR overlap extension, as previously described by Ho et al. (18). The 5' half-clone of SIVmac239, pVP-1 (33), was used as the template for amplification of the half-fragments for the overlap extension PCR with primers 239-2459 (primers used for site-directed mutagenesis are in Table 1) and 239-K65R (R) for the 5' amplicon and 239-K65R (F) and SIV-RT8 (R) for the 3' amplicon. Both reactions were carried out using Promega *Pfu* under recommended reaction conditions (Promega, Madison, Wis.) and thermal cycling conditions as follows: initial denaturation at 94°C for 75 s followed by 30 cycles of 94°C for 45 s, 53°C for 30 s, and 72°C for 150 s, followed by a single extension cycle of 72°C for 12 min. Products were separated by electrophoresis on a 1.3% agarose gel, and the 5' and 3' amplicons were separately extracted from the gel by using the QIAEX gel extraction kit (Qiagen, Valencia, Calif.). Aliquots (1 μl) of each half-fragment were used for the overlap extension phase to produce a 965-bp fragment of SIVmac239 containing the K65R mutation. This reaction was carried out using primers 239-2459 and SIV-

TABLE 3. Genotypes of the first 195 codons of RT from selections with PMPA alone^a

Virus and selection ^d	Genotype found after passage no.						
	1	2	3	4	5	6	7
SIVmac239 (WT)^c							
Selection 1	+	+	+	+	K65R	—	
Selection 2	+	+	+	WT	WT	—	
Selection 3	+	+	+	+	WT	—	
Selection 4	+	+	+	WT	—		
Selection 5	+	+	+	M164I ^b	—		
Selection 6	+	+	+	K65R ^b	K65R ^b	—	
SIVmac239-184V							
Selection 1	+	+	+	K70N	K70N	—	
Selection 2	+	+	+	+	WT	—	
Selection 3	+	+	+	+	—		
Selection 4	+	+	+	WT ^b	K65R ^b	K65R	—
						I118V ^b	
Selection 5	+	+	+	K65K/R ^b	K65R ^b	K65R ^b	—
Selection 6	+	+	+	WT ^b	—		

^a The detection of virus replication is designated by + or by the genotype that was determined by sequencing. Selections 1 to 3 were carried out under the first set of conditions, and selections 4 to 6 were carried out under the second set of conditions as described above (Materials and Methods). —, no virus replication was detected by p27 ELISA.

^b A titer of 10,000 focus-forming units was used to start the selection, as opposed to the 1,000 focus-forming units otherwise used.

^c WT, wild type.

^d The concentrations (in micromolars) of PMPA used were as follows: passage 1, 5; passage 2, 10; passage 3, 20; passage 4, 40; passage 5, 80; passage 6, 160; and passage 7, 320.

RT8 (R), Promega *Pfu*, and thermal cycling conditions as described above. The reaction mix was processed through a Microcon YM-50 column (Millipore, Bedford, Mass.), and the eluate was added to a 20- μ l reaction mix containing a final concentration of 2 U of GIBCO *Taq* polymerase, GIBCO reaction buffer, and 200 nM dATP (GIBCO). This mix was incubated at 72°C for 20 min to add poly(A) to the 3' end of the amplicon. This fragment was then cloned into pCRII TA plasmid from the Invitrogen cloning kit to produce pCRII-K65R. The correct sequence of RT and the presence of K65R were confirmed by DNA sequence analyses. However, because of problems that we encountered in attempts to clone this fragment into the 5' half-plasmid (pVP-1) using the *Pvu*II site, we performed another overlap extension of this fragment to extend it to the *Bam*HI site. Two amplicons were prepared for the overlap extension phase of PCR: reaction 1 using pVP-1 as a template and forward primer 239-*Bam*HI and reverse primer 239-2565 (R) and reaction 2 using pCRII-K65R as a template and forward primer 239-2459 and reverse primer SIV-RT8 (R). Both reactions were carried out with Platinum High-Fidelity polymerase (GIBCO) under recommended conditions and thermal cycling conditions as follows: initial denaturation at 94°C for 40 s followed by 30 cycles of 94°C for 30 s, 53°C for 30 s, and 68°C for

120 s, and a single extension cycle of 72°C for 5 min to complete the reaction. Products were separated on a 1.3% agarose gel and extracted as outlined above. Overlap extension was performed with aliquots (1 μ l) of each of the two gel-extracted half-fragments, primers 239-*Bam*HI and SIV-RT8 (R), and Platinum High-Fidelity Polymerase (GIBCO), under recommended conditions and thermal cycling conditions as follows: initial denaturation at 94°C for 40 s followed by 35 cycles of 94°C for 40 s, 53°C for 30 s, and 68°C for 120 s and a single extension cycle of 72°C for 5 min to complete the reaction. A 1,564-bp product was obtained by electrophoresis in a 1.3% agarose gel, and poly(A) was added as described above. This fragment was then cloned into pGEM-T (Promega). A *Bam*HI-*Bsu*361 fragment from this was subcloned into pVP-SS-wt (the *Spe*I-to-*Sph*I fragment of SIVmac239 cloned into pLitmus38 [New England Biolabs, Beverly, Mass.]). The *Spe*I-to-*Sph*I fragment of this subclone was then used to replace the wild-type fragment in pVP-1. DNA sequence analysis of the resulting clone was performed to confirm the sequence integrity of RT and the presence of the K65R mutation.

Animals and sample collection. Juvenile rhesus macaques (*Macaca mulatta*) were from the type D-retrovirus- and SIV-free colony at the California National Primate Research Center. Animals were 6 to 8 months of age (~1.2 to 2.1 kg). We strictly adhered to the Guide for the Care and Use of Laboratory Animals prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (38). When necessary, animals were immobilized with 10 mg of ketamine-HCl (Parke-Davis, Morris Plains, N.J.) per kg of body weight injected intramuscularly. EDTA-anticoagulated blood samples were collected to measure viral and immunologic parameters. Complete blood counts were performed on EDTA-anticoagulated blood samples. Samples were analyzed by using an automated electronic cell counter (Baker 9000; Serono Baker Diagnostics), and differential counts were determined manually. Animals were euthanatized when it became apparent that their condition was terminal, according to criteria described previously (59).

Virus inoculation. Animals were inoculated intravenously with 0.5 ml of virus dilution containing 10³ 50% tissue culture infective doses of either SIVmac239 or SIVmac239-184V as previously described (58).

Administration of drugs. Stock solutions of (–)-FTC (16 mg/ml) were prepared in phosphate-buffered saline (pH 7.4; Sigma-Aldrich). PMPA was suspended in distilled water (60 mg/ml) with NaOH added to a final pH of 7.0. Both (–)-FTC and PMPA stocks were filter sterilized (pore size, 0.2 μ m; Nalgene, Rochester, N.Y.) and were stored at 4°C. Drugs were administered subcutaneously into the backs of animals at a regimen of 8 mg per kg once daily for (–)-FTC and 10 mg per kg once daily for PMPA. Drug dosages were adjusted weekly according to body weight.

Viral RNA levels in plasma. Taqman real-time PCR was used to quantitate SIVmac239 RNA as previously described (29). This assay has a sensitivity of 50 copies of viral RNA per ml of plasma (29).

Lymphocyte phenotyping by three-color flow cytometry. T-lymphocyte antigens were detected by direct labeling of whole blood with peridinin chlorophyll protein-conjugated anti-human CD8 (clone SK1; Becton Dickinson Immunocytometry Inc., San Jose, Calif.), phycoerythrin-conjugated anti-human CD4 (clone M-T477; Pharmingen), and fluorescein-conjugated anti-human CD3 (clone SP34; Pharmingen). A separate aliquot of blood was labeled with fluorescein-conjugated anti-human CD3 and PerCP-conjugated anti-human CD20 (clone

TABLE 4. Genotypes of the first 195 codons of RT from selections with 3TC or in passages without drug^a

Virus and selection	Genotype after each passage									
	1	2	3	4	5	6	7	8	9	10
SIVmac239 (WT)^b										
Selection 1	WT	WT	WT	M184I	M184I	M184I	+	M184I	M184I	
Selection 2	WT	WT	WT	M184I	M184I	M184I	+	M184I/V	M184I/V	
Selection 3	WT	WT	WT	M184I	M184I	M184I	+	M184I	M184I	
SIVmac239-M184V										
Selection 1	+	+	+	+	M184V	+	M184V	M184M/V	M184M/V	WT
Selection 2	+	+	+	+	M184V	+	+	+	+	M184V
Selection 3	+	+	+	+	M184V	+	+	+	+	M184V

^a The detection of virus replication is designated by + or by the genotype that was determined by sequencing. Selections were carried out according to the second set of conditions as described above (Materials and Methods). SIVmac239-M184V was passaged without drug. SIVmac239 (WT) was passaged at the following 3TC concentrations (in micromolars): passage 1, 1; passage 2, 2; passage 3, 4; passage 4, 8; passage 5, 16; passage 6, 32; passage 7, 64; passage 8, 128; passage 9, 256.

^b WT, wild type.

TABLE 5. Antiviral susceptibilities of SIVmac239 site-directed mutants expressing 3TC- or PMPA-associated resistance mutations in RT and of drug-resistant isolates from selections with PMPA or with 3TC plus PMPA

Initial virus or mutation	Selection conditions	Round	Mutation(s) in RT	Results for drug used			
				PMPA		3TC	
				Mean EC ₅₀ (μ M) $\pm$ SEM ^a	Resistance (<i>n</i> -fold) ^b	Mean EC ₅₀ (μ M) $\pm$ SEM ^a	Resistance (<i>n</i> -fold) ^b
SIVmac239			WT ^c	3.7 $\pm$ 0.39	1.0	1.2 $\pm$ 0.12	1.0
SIVmac239-184V			M184V	2.1 $\pm$ 0.22	0.56	>500	>400
SIVmac239-65R			K65R	14 $\pm$ 1.3	3.8	150 $\pm$ 19	130
M184V	3TC/PMPA	5	K65R, R82K/R, I118I/V, M184V	7.3 $\pm$ 0.56	2.0	ND	ND
M184V	3TC/PMPA	6	K65R, N69S, I118V, V181I	27 $\pm$ 1.8	7.3	>500	>400
M184V	PMPA	6	K65R, I118V	21 $\pm$ 3.6	5.7	440 $\pm$ 55	370
M184V	High 3TC/PMPA	3	K70T, M184V	3.1 $\pm$ 0.34	0.83	ND	ND
M184V	High 3TC/PMPA	3	K65R, M184V	5.2 $\pm$ 0.81	1.4	ND	ND

^a Values are from three or more experiments, with four determinations per experiment.

^b Increase over value for SIVmac239. Values less than 1.0 represent increased susceptibility. ND, not determined.

^c WT, wild type.

L27; Becton Dickinson). Red blood cells were lysed, and the samples were fixed in paraformaldehyde using the Coulter Q-prep system (Coulter Corp., Hialeah, Fla.). Lymphocytes were gated by forward and side light scatter and were then analyzed with a FACScan flow cytometer (Becton Dickinson). CD4⁺ T lymphocytes and CD8⁺ T lymphocytes were defined as CD3⁺ CD4⁺ and CD3⁺ CD8⁺ lymphocyte populations, respectively. B lymphocytes were defined as CD3⁻ CD20⁺ lymphocytes.

RESULTS

SIV variants resistant to the combination of 3TC plus PMPA. Viruses resistant to the combination of 3TC and PMPA were selected by serial passages of wild-type SIVmac239 in increasing concentrations of these two drugs. Drug concentrations for the first round of selection were approximately the EC₅₀ for each drug, and concentrations of each drug were doubled with each successive round of selection. Results from these selections are shown in Table 2. Three of six selections with wild-type SIV in 3TC plus PMPA yielded virus replication in the fifth passage (with concentrations of 16 μ M 3TC and 80 μ M PMPA). The selections that yielded virus in the fifth passage were characterized by DNA sequence analysis of the first 195 codons of the RT-encoding region of the *pol* gene. Each virus capable of replication in the fifth passage showed the K65R genotype but, surprisingly, did not have M184V or any other mutations in the region of RT that were sequenced.

In the selections of wild-type virus with PMPA alone, the K65R mutation was present in proviral DNA from two of the six selections, four of which yielded virus in the fifth passage (Table 3). Thus, selection of the 65R mutation with 3TC plus PMPA was not significantly different from the selection with PMPA alone. No other mutations were found in the RT-encoding region of virus from these selections, with the exception of an M164I mutation, which appeared in the fourth passage (Table 3, selection 5). Virus from this selection failed to show replication in the fifth passage.

Results presented in Table 4 show that, starting from wild-type SIVmac239, SIV variants with resistance to 3TC alone developed quickly, as reported previously (4). By the end of the fourth passage (8 μ M 3TC), the M184I genotype had developed in all three of these selections. This genotype was consistently maintained throughout four more passages of in-

creased selection pressure, although a mixture of M184V and M184I genotypes was detected in one of these three selections by the eighth passage (128 μ M 3TC; Table 4, upper portion, selection 2).

To confirm the role of K65R in SIV resistance to 3TC and PMPA, we introduced this mutation into the molecular clone SIVmac239 by using site-directed mutagenesis. The K65R mutant displayed 3.8-fold resistance to PMPA (Table 5), with an EC₅₀ of 3.7 μ M for SIVmac239 and 14 μ M for SIVmac239-65R (Fig. 1A). By contrast, a site-directed mutant with M184V displayed 1.8-fold hypersensitivity (0.56-fold resistance) to

FIG. 1. Inhibition of SIVmac239 (Δ), SIVmac239-65R ($\circ$), or SIVmac239-184V ($\blacksquare$) by PMPA (A) or 3TC (B). Results are from at least three experiments with four determinations per experiment. Bars represent standard errors of the means.

TABLE 6. Genotypes of the first 195 codons of RT from selection of SIVmac239-K65R with 3TC and PMPA or with 3TC alone^a

Virus and selection	Genotype found after each passage:					
	1	2	3	4	5	6
SIVmac239-K65R						
Selection 1	+	+	+	K65R	K65R	—
Selection 2	+	+	+	K65R R82K	K65R R82K M184V	K65R R82K M184V
Selection 3	+	+	+	K65R	K65R M184V	K65R M184V
SIVmac239-K65R						
Selection 1	+	+	+	+	K65R	K65R M184V
Selection 2	+	+	+	+	K65R D67N M184M/V	K65R D67N M184V
Selection 3	+	+	+	+	R35R/K K65R R82R/K	R35K K65R R82K

^a The detection of virus replication is represented by a + or by the genotype that was determined by sequencing. Selections were carried out according to the second set of conditions as described above (Materials and Methods). —, no virus replication was detected by p27 ELISA. For the first set of selections, the concentrations of 3TC to PMPA (in micromolars) were as follows: passage 1, 10 and 20; passage 2, 20 and 20; passage 3, 40 and 20; passage 4, 80 and 20; passage 5, 160 and 20; and passage 6, 320 and 20. The concentrations of 3TC alone used for the second set of selections were as follows: passage 1, 10; passage 2, 20; passage 3, 40; passage 4, 80; passage 5, 160; and passage 6, 320.

PMPA (Table 5) with an EC₅₀ of 2.1 μ M (Fig. 1A). The role of M184V in SIV resistance to 3TC has previously been demonstrated (4), and the M184V mutant showed >400-fold resistance to 3TC (Fig. 1B), with EC₅₀s of 1.2 μ M for SIVmac239 and >500 μ M for SIVmac239-184V. The K65R mutant displayed 130-fold resistance to 3TC, with an EC₅₀ of 150 μ M (Fig. 1B). This was higher than the 20- to 40-fold resistance to 3TC that has previously been reported for K65R mutants of HIV-1 (2, 14, 16, 64).

Reversion of M184V. To further investigate the effect of selection with 3TC plus PMPA on the M184V mutation, a site-directed mutant of SIVmac239 containing M184V (SIVmac239-184V) was passaged in 3TC plus PMPA in the same manner as described above. Table 2 shows that, under these conditions, four of the six selections had virus replication in the fifth passage (16 μ M 3TC plus 80 μ M PMPA). DNA sequence analysis of samples from these revealed that all four had the K65R mutation (Table 2). Interestingly, the M184V mutation was present only in virus from one of these selections. In three of the four selections that yielded virus at passage 5, the M184V mutation reverted, despite the presence of 3TC. In two of the selections at the fifth passage, no mutations other than K65R were found in the first 195 codons of RT (Table 2, middle portion, selections 2 and 3). Virus from a third selection was found to have K65R, N69S, and V181I in the fifth passage (Table 2, middle portion, selection 6). When this latter selection also yielded virus during a sixth passage of selection (160 μ M PMPA and 32 μ M 3TC), the complete RT-encoding region was sequenced and an additional I118V mutation was found. Virus from this sixth passage of selection with K65R, N69S, I118V, and V181I was tested by FIA for phenotypic resistance to PMPA or to 3TC and was found to be 7.3-fold

resistant to PMPA and >400-fold resistant to 3TC (Table 5). This level of 3TC resistance is higher than that obtained with SIVmac239-65R. These data suggest that one or more of the mutations present along with K65R increase the level of 3TC resistance. Insertion mutations near position 69 (65) and a V118I mutation in HIV-1 RT (17) are known to contribute to 3TC resistance.

Variants of SIVmac239-184V expressing resistance to PMPA were also selected in the absence of 3TC. The M184V mutation was not present in any of the samples sequenced from these selections (Table 3). Two of the four selections that yielded virus replication in the fifth passage (80 μ M PMPA) contained the K65R mutation. Both selections with K65R continued to replicate in a sixth passage (160 μ M PMPA), and one of these developed an additional I118V mutation. Subsequent phenotypic analysis showed that the isolate containing both K65R and I118V was 5.7-fold resistant to PMPA and 370-fold resistant to 3TC (Table 5).

As an additional control, SIVmac239-184V was passaged in the absence of drug. Under these conditions, virus replication was rapid and the mutant genotype was stable throughout seven passages (Table 4). Two of the three selections maintained the M184V mutation through 10 passages without drug, while in one of the selections a mixture of methionine and valine at codon 184 was detected during the eighth passage (Table 4, lower portion, selection 1). By the 10th round, only the wild-type methionine was detected. These data demonstrate that reversion of M184V in SIV is greatly enhanced by PMPA.

The M184V mutation impedes development of PMPA resistance. In order to examine the effects of M184V on the development of resistance to PMPA, selections were carried out under conditions expected to prevent reversion of M184V. For these selections, the SIVmac239-184V mutant was passaged in the presence of high levels of 3TC (100 μ M) and increasing levels of PMPA. Genotypic analysis of proviral DNA from the third passage (100 μ M 3TC plus 20 μ M PMPA) showed that in three of the five selections the M184V and K65R mutations were both present (Table 2, bottom portion). These variants with both M184V and K65R were only 1.4-fold resistant to PMPA (Table 5), compared to being 3.8-fold resistant to PMPA for virus with K65R alone.

Although K65R did develop in three of the five selections that replicated in the third round, virus replication was rarely observed at levels of PMPA greater than 20 μ M (Table 2). Only one of the six selections with 100 μ M 3TC yielded detectable virus in the presence of 40 μ M PMPA (fourth round). Virus from this selection also showed replication at 80 μ M PMPA plus 100 μ M 3TC (fifth round) and developed an R82K mutation in addition to K65R and M184V. This is in contrast to the SIVmac239-184V selections described above (with gradually increasing levels of both 3TC and PMPA) in which virus replication was evident in four of the six selections at 80 μ M PMPA plus 16 μ M 3TC. Thus, the maintenance of the M184V mutation not only diminished the effect of K65R on phenotypic resistance to PMPA (as observed previously with HIV [64]) but also decreased the number of selections in which virus was capable of replicating in higher levels of PMPA.

Resistance of K65R mutants of SIV to 3TC. We also evaluated the effect of the K65R mutation on resistance to the

FIG. 2. Virus levels in juvenile macaques infected with SIVmac239 (group A, dashed line) or SIVmac239-184V (group B, solid line). (A) Levels of SIV RNA in plasma from individual animals following the initiation of PMPA therapy. SIV RNA levels in plasma were determined by real time RT-PCR. Group B animals received (–)-FTC beginning 1 day before inoculation and continuing throughout the experiment. Both groups received PMPA therapy starting at 46 weeks after virus inoculation. Animal no. 31339 was euthanized because of simian AIDS 11 weeks after PMPA therapy began. (B) Average viral RNA levels for group A and group B animals. Average values for group A are also shown without animal 31339, because this animal was already nearing terminal stages of disease when PMPA therapy began.

combination of 3TC plus PMPA. For these selections, SIVmac239-65R was passaged in 3TC plus PMPA. The first round of selection was done in the presence of 10 μ M 3TC and 20 μ M PMPA. In each successive round of selection, the 3TC concentration was increased twofold and the PMPA concentration was kept constant at 20 μ M. Through the fourth round of selection (80 μ M 3TC plus 20 μ M PMPA), K65R remained and there were no mutations at codon 184 (Table 6). Virus from one of these cultures also had the R82K mutation. By the fifth round of selection (160 μ M 3TC plus 20 μ M PMPA), the M184V mutation was present in virus from two of these selections (Table 6, selections 2 and 3). These results demonstrate that selection of the M184V mutation in the presence of PMPA requires extremely high levels of 3TC; these levels may be difficult to achieve without adverse effects in vivo.

Similar results were obtained from selections with SIV-

mac239-65R in the presence of increasing levels of 3TC alone. The K65R mutation did not revert, and the M184V mutation did not emerge until the sixth round (320 μ M 3TC) in two of three selections (Table 6, selections 1 and 2). The K65R mutation appeared to enable replication at unusually high levels of 3TC. In the third selection, the M184V mutation did not arise but R82K and R35K mutations appeared (Table 6, selection 3).

We analyzed 3TC resistance in selections with two PMPA-resistant variants of SIVmac251 that had been obtained from PMPA-treated rhesus macaques (57, 60). These two viruses were each used in selections with a constant level of PMPA (20 μ M) and sharply increasing levels of 3TC (beginning at 10 μ M and moving to 25, 100, and 1,000 μ M in subsequent rounds). Both viruses developed mutations in codon 184 (data not shown). By the fourth passage (1,000 μ M 3TC), SIVmac385

TABLE 7. Genotypes of the RT region of SIV isolates from animals receiving PMPA (group A) or FTC plus PMPA (group B)^a

Group	Animal no.	Genotype or other result after PMPA treatment began (no. of wk later)				
		0	8	13	24	39
A	31304	WT	ND	K65R, Y162N	K65R, N69S, Y162N ^b	ND
	31339	WT	K65R	Necropsy at 11 wk		
	31632	WT	R35K, K65R	R35K, K65R, N69S	R35K, K65R, N69S ^b	K65R, N69S
B	31579	M184V	ND	K65R, I118V, M184V	K65R, I118V ^b	ND
	31585	M184V	K65R, R82K, M184V	K65R, R82K, M184M/V	K65R, N69S, R82K ^b	K64R, K65R, N69S
	31690	M184V	M184V	K65R	K65R ^b	ND

^a DNA sequences were determined by using proviral DNA extracted from CEMx174 cells infected with virus isolated from SIV-infected animals (Materials and Methods).

^b RT sequences were determined by using proviral DNA as indicated above and by using viral RNA that was extracted from animal plasma and amplified with RT-PCR (Materials and Methods). In each of the three cases indicated, both sequencing methods yielded identical results. ND, not determined.

had developed M184I and SIVmac055 had developed M184V. SIVmac385 with M184I was passaged in 500 μ M 3TC and 12.5 μ M PMPA eight additional times. Throughout that time, the M184I genotype was maintained and M184V did not appear.

Reversion of M184V in vivo. We examined the effect of PMPA therapy on the stability of the M184V mutation in rhesus macaques that had been infected with SIVmac239-184V. For these studies we used juvenile macaques that had been infected with SIVmac239 or SIVmac239-184V for 46 weeks as part of another study (58). Animals in group A ($n = 3$) were infected with SIVmac239, and those in group B ($n = 3$) were infected with SIVmac239-184V. Animals in group B were treated with (–)-FTC (8 mg/kg given once daily beginning 1 day prior to virus inoculation) in order to prevent reversion of M184V (58). (–)-FTC is an analog of 3TC with stronger in vitro activity and an improved pharmacokinetic profile (12, 13). Both groups became persistently infected and had similar viral RNA levels from 2 weeks after inoculation until 46 weeks when PMPA was started in both groups (10 mg/kg, administered subcutaneously). (–)-FTC treatment was also continued in animals of group B. The dose of PMPA that was used in this experiment is smaller than the dose that is typically used in rhesus macaque studies (30 mg/kg/day) (30, 40, 56, 57). We chose this dose of PMPA in an attempt to also evaluate whether the M184V mutation of SIV would confer hypersensitivity to PMPA in vivo.

All animals showed a decline in viral RNA levels after the administration of PMPA, with the exception of animal 31339 of group A (Fig. 2A). Although the levels of (–)-FTC used in this study did not previously reduce virus loads in SIVmac239-184V-infected animals (58), the average viral RNA levels in this study were consistently lower in group B animals from weeks 2 through 8 (Fig. 2B). This suggests that the M184V mutation may confer hypersensitivity to PMPA in vivo. However, this difference was not statistically significant ($P = 0.09$ at week 4, $P = 0.23$ at week 6, and $P = 0.07$ at week 8) and a larger study will be necessary to confirm this. Viral loads were not affected by PMPA administration in animal no. 31339, which progressed to terminal stages of disease soon after PMPA therapy began and was euthanatized after signs of simian AIDS were detected at 11 weeks. For this reason, the average virus levels are represented both with and without animal no. 31339 in group A (Fig. 2B). As indicated by Fig. 2A, virus levels in each animal returned to nearly the pre-PMPA level within 35 weeks of PMPA administration.

Genotypic analysis of virus from the animals with SIVmac239-184V (group B) revealed reversion of this mutation to the wild-type codon (184M) in each of the animals. The M184V mutation was still present, and no 184M revertants were detected at 8 weeks after initiation of PMPA-plus-(–)-FTC therapy (Table 7). However 184M was predominant from 13 weeks onward in one animal (no. 31690) and from 24 weeks onward in the other two animals. The K65R mutation appeared prior to or concomitant with reversion of M184V in each animal (Table 7). The K65R mutation was present in all subsequent isolates from these animals. Other mutations that appeared in virus isolates from group B animals were I118V from one animal (no. 31579) and N69S, R82K, and K64R in another (no. 31585). Each of these mutations has been identified previously during studies with PMPA monotherapy of SIV-infected macaques (57, 60). As expected, the K65R mutation appeared in all three animals with SIVmac239 that were treated with PMPA alone (Table 7). Phenotypic analysis of virus isolates collected 39 weeks after initiation of PMPA therapy from both groups of animals showed that these isolates had between 90- and 300-fold resistance to 3TC and between two- and sixfold resistance to PMPA.

Absolute counts of CD4⁺ T lymphocytes and CD8⁺ T lymphocytes in peripheral blood showed wide variation over time. However, in each animal of both experimental groups, these populations, as well as the total lymphocyte counts, briefly increased immediately after the initiation of PMPA therapy (data not shown). Although PMPA therapy did not clearly affect the absolute number of CD4⁺ T lymphocytes in each group beyond the brief initial increase (Fig. 3), the percentage of all peripheral blood lymphocytes that were CD4⁺ CD3⁺ did show an increasing trend in both groups (Fig. 3). Additionally, the CD4⁺ to CD8⁺-T-lymphocyte ratio rose significantly more highly in group B than in group A animals after 2 weeks of PMPA therapy ($P = 0.025$; two-tailed t test) (Fig. 3). By 8 weeks of PMPA therapy, there were no longer any significant differences between the two groups in any of these parameters.

DISCUSSION

The M184V mutation in RT of HIV-1 emerges rapidly in AIDS patients who are treated with 3TC and is the predominant determinant of high-level resistance to 3TC or (–)-FTC. However, M184V may contribute to the long-term success of some drug combinations because this mutation can cause phe-

FIG. 3. Changes in absolute CD4⁺-T-lymphocyte counts, percentage of CD4⁺ CD3⁺ cells among total lymphocytes, and CD4⁺-to-CD8⁺-T-lymphocyte ratios. Animals were inoculated intravenously with SIVmac239 (dashed lines) or SIVmac239-184V (solid lines). SIVmac239-184V-infected animals were treated with (–)-FTC beginning 1 day before inoculation, and both groups of animals were treated with PMPA starting 46 weeks after virus inoculation. Values are presented for the first 12 weeks of PMPA therapy. Animal no. 31339 was euthanatized because of simian AIDS 11 weeks after PMPA therapy began.

notypic suppression of certain mutations that are responsible for resistance to other drugs. For example, the addition of M184V to a virus with AZT resistance mutations in RT restores phenotypic susceptibility to AZT (28) and additional mutations are required to confer coresistance to both drugs (39). Another beneficial effect of M184V may result from the hypersensitivity of these variants to certain other antiviral drugs. The M184V mutation of HIV-1 is known to render virus

more susceptible to inhibition by the nucleotide analogs adefovir and PMPA (35, 64). The M184V mutation in RT of SIV similarly confers high-level resistance to 3TC (4) and results in hypersensitivity to PMPA. The goal of the work reported here was to evaluate the effect of M184V on resistance to the combination of 3TC plus PMPA.

We have found that the M184V mutation of SIV reverts to the wild-type codon (184M) in selections with PMPA, even when 3TC is present. Moreover, this reversion occurs both in vitro and in vivo. In the cell culture system, selection with PMPA or 3TC plus PMPA consistently yielded SIV with the wild-type codon (methionine) at position 184, even when the selections were started with SIV containing the M184V mutation. In contrast, the M184V mutation was quite stable in the absence of PMPA. No reversion of M184V was observed through seven passages of SIVmac239-184V in CEMx174 cells in the absence of drugs. Through 10 passages, reversion of M184V was observed in only one of three cultures. Thus, reversion of M184V is greatly enhanced by PMPA. It was recently shown that reversion of the M184V mutation in HIV-1 is enhanced by AZT (8). This reversion occurred in the presence of AZT alone or of AZT plus low levels of 3TC but not in the presence of AZT and a higher concentration of 3TC (0.25 μ M or greater). These effects of AZT and our results with PMPA provide two examples of therapeutic strategies that may be able to select for loss of a resistance-conferring mutation.

The M184V mutation of SIVmac239-184V similarly reverted in virus populations from each of three macaques that were treated with (–)-FTC plus PMPA. These animals had been infected and treated with (–)-FTC monotherapy for 46 weeks prior to the (–)-FTC-plus-PMPA therapy, and during that time there was no reversion of the M184V mutation (58). However, following initiation of therapy with (–)-FTC plus PMPA, the M184V mutation reverted to 184 M in virus isolates from each of the three animals. The K65R mutation, which is known to confer PMPA resistance in SIV (57), arose before or concomitant with reversion of M184V and was able to confer resistance to 3TC plus PMPA. These observations could be explained by reduced fitness of the K65R/M184V double mutant relative to the K65R mutant or by reduced fitness of M184V in the presence of PMPA. The timing of the appearance of the 65R and 184M genotypes coincides with the disappearance of the difference between average virus levels of the two groups. Isolates from both groups and a site-directed mutant of SIVmac239 containing K65R were >100-fold resistant to 3TC. Thus, K65R alone is able to confer reduced susceptibility to the combination of 3TC plus PMPA. This level of resistance to 3TC is somewhat higher than the level of 3TC resistance conferred by K65R in HIV-1, which was reported to be 20- to 40-fold (2, 14, 16, 64). These studies in the animal model support our in vitro results that PMPA treatment selects for reversion of M184V in SIV RT.

In our studies with the cell culture system, we found that the presence of the M184V mutation significantly impeded the emergence of PMPA resistance. In five of seven selections in which M184V was maintained, virus replication did not occur at levels of PMPA higher than 20 μ M (third passage of selection) (Table 2, midsection, selection 5; and Table 2, bottom section, selections 1, 3, 5, and 6). This effect could also be explained by reduced fitness of a K65R/M184V double mutant.

By contrast, in selections with virus that had the wild-type methionine at codon 184, there was detectable replication through at least the fifth passage (80 μ M PMPA). Thus, phenotypic PMPA resistance is much more difficult to achieve in vitro in SIV with the M184V mutation. When selections were initiated with SIV containing the K65R mutation, it was possible to select virus containing both the K65R and M184V mutations. However, these did not emerge until a very high level of 3TC (greater than 80 times the EC_{50}) was reached in the selection process. These data suggest that it is unlikely that levels of 3TC adequate for maintenance of the M184V mutation in SIV RT can be achieved in vivo.

Several features of these studies with 3TC plus PMPA could have important implications for the use of this combination in AIDS therapy if HIV-1 is similar to SIV in resistance profiles with these two drugs. The rapid reversion of M184V in the presence of PMPA or 3TC plus PMPA is consistent with the hypersensitivity of M184 variants to PMPA, which was recently reported in patients treated with an oral prodrug of PMPA (PMPA DF) who had HIV-1 expressing the M184V mutation (34). This hypersensitivity to PMPA could be advantageous during use of the 3TC-plus-PMPA combination, although this effect would be transient if M184V reversion occurs rapidly in HIV-1 as it does with SIV. Moreover, the PMPA-induced reversion of M184V that we observed coincident with the development of K65R may alter the phenotypic resistance to other drugs. Drug susceptibility assays indicate that K65R reduces phenotypic resistance to AZT in a manner similar to the reduction that M184V causes (2). On the other hand, the K65R mutation in HIV-1 confers some cross-resistance to several other drugs, including 3TC, 2',3'-dideoxyinosine, 2',3'-dideoxycytidine, and abacavir (14, 54). The clinical relevance of these phenotypes remains to be established. We are presently evaluating the combination of 3TC plus PMPA and the roles of K65R and M184V on profiles of resistance of HIV-1.

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