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Mutations at Codon 184 in Simian Immunodeficiency Virus Reverse Transcriptase Confer Resistance to the (−) Enantiomer of 2′,3′-Dideoxy-3′-Thiacytidine

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Variants of simian immunodeficiency virus (SIV) that display greater than 2,000-fold resistance to the (−) enantiomer of 2′,3′-dideoxy-3′-thiacytidine (3TC) were generated through in vitro passage and drug selection. The polymerase regions of several of these resistant viruses were sequenced and were found to share either of two codon alterations at site 184 in reverse transcriptase (ATG to ATA [methionine to isoleucine] and ATG to GTA [methionine to valine]). The biological relevance of these substitutions for 3TC was confirmed by site-directed mutagenesis with the SIVmac239 infectious recombinant clone of SIV.

Reverse transcriptase (RT) plays a key role in the human immunodeficiency virus (HIV) type 1 (HIV-1) life cycle and is therefore an important target of antiviral drugs (16). However, the error-prone nature of this enzyme results in mutations in RT that are associated with drug resistance (13, 22). Drug-resistant variants of HIV have been isolated from patients undergoing prolonged therapy and by tissue culture selection in the presence of increasing concentrations of antiviral compounds (6, 12).

Both an M184I substitution and an M184V substitution in the RT of HIV-1 have been shown to confer high-level resistance (up to 1,000 times) to the (−) enantiomer of 2′,3′-dideoxy-3′-thiacytidine (3TC) as well as low-level resistance (3- to 10-fold) to each of 2′,3′-dideoxyinosine (ddI) and 2′,3′-dideoxycytidine (ddC) (3, 7, 19, 23). In clinical studies, resistance to 3TC was observed in nearly all patients who received 3TC monotherapy for more than 12 weeks (20).

In view of the need for animal models to study antiviral drugs, we were interested in determining the effects of 3TC on simian immunodeficiency virus (SIV) replication. We used the technique of in vitro passage and drug selection to generate SIV variants resistant to 3TC.

Drugs. 3TC was a gift of BioChem Pharma Inc., Montreal, Quebec, Canada. 3′-Azido-3′-deoxythymidine (AZT), ddI, and ddC were obtained from Glaxo-Wellcome Inc. (Research Triangle Park, N.C.), Bristol-Myers Squibb (Wallingford, Conn.), and Sigma Chemical Co. (St. Louis, Mo.), respectively. 2′,3′-Didehydro-2′,3′-dideoxythymidine (d4T), nevirapine, indinavir, and saquinavir were gifts of Bristol-Myers Squibb, Boehringer-Ingelheim Inc. (Ridgefield, Conn.), Merck Inc. (West Point, Pa.), and Roche Inc. (Basel, Switzerland), respectively.

Cells and viruses. The human $CD4^+$ T-cell line C8166 (European Collection of Animal Cell Cultures; Public Health Laboratory Service, London, United Kingdom) was grown in RPMI 1640 medium (Gibco-BRL Laboratories, Mississauga, Ontario, Canada) supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories, Toronto, Ontario, Canada), 2 mM L-glutamine, 100 U of penicillin G per ml, and 100 μg of streptomycin per ml. These cells were used for all studies. Two full-length viruses cloned by molecular biology-based techniques, SIVmac32H(pJ5) and SIVmac32H(pC8), were used in the initial drug selection experiments. These viruses differ in the *nef* coding region by a naturally occurring in-frame deletion of four amino acids in pC8 and two conservative amino acid changes (18).

Selection for resistance. C8166 cells (10^6 cells) were infected for 2 h with 200 50% tissue culture infective doses of either the pJ5 or pC8 151V viral stocks. The cells were then washed extensively and maintained in tissue culture medium in either the absence or the presence of subinhibitory concentrations of 3TC, beginning with 0.01 μM. The medium was changed twice weekly; each replacement contained an increased drug concentration, as follows: 0.01, 0.05, 0.2, 1, 2.5, 10, 25, 100, 250, 500, and 750 μM. Culture fluids (0.5 ml) from each passage were used to infect fresh C8166 cells as described previously (7). Cultures were monitored for the presence of RT activity and the presence of a cytopathic effect as described previously (9). After 8 and 24 weeks (final 3TC concentration, 2.5 or 750

TABLE 1. Selection of SIV variants resistant to 3TC

Virus	No. of wk in passage[a]	Highest concn (μM) of 3TC attained[b]	Mutations found by sequencing[c]	IC_{50} (μM) of 3TC[d]
SIVmac32H(pJ5)	0	0		0.13 ± 0.04
	8	2.5	M184I	>500
	24	750	M184V	>2,000
SIVmac32H(pC8)	0	0		0.45 ± 0.01
	8	2.5	M184I	>500
	24	750	M184V	>2,000

[a] All cultures were passaged twice weekly.
[b] Whenever cultures became positive for cytopathic effect and RT activity, the drug concentrations were gradually increased.
[c] Substitutions were detected by cloning and sequencing as described in the text.
[d] Results were calculated on the basis of RT activity in culture fluids. Data are means ± standard deviations for three replicate samples.

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TABLE 2. Oligonucleotides used for cloning and sequencing

Oligonucleotide	Sequence (5′→3′)	SIVmac32H coordinates
SRT01	GGGGATGTCTTTAAACC	3099–3115
SRT02	GCTGGCTCTATCTTTTCC	4817–4800
SRT03	GAACAAATGGAGAATGC	3321–3337
SRT04	GCAGAGCCAGGAAAGCG	3529–3545
SRT05	CTCTTGAATAGCATAGGG	3739–3756
SRT06	GGAGTATTAAATTGGGC	3901–3917
SRT07	GGTCTTATAAAATTCACCA	4127–4145
SRT08	GGCAGGTAACCTGGATACC	4331–4349
SRT09	CTACTAATCAACAAGCAG	4529–4546

μM), viral variants of pJ5 and pC8 contained either the M184I or M184V substitutions, respectively, and were capable of growth in the presence of greater than 500 and 2,000 μM 3TC, i.e., more than 1,000- and 4,000-fold the usual inhibitory drug concentration, respectively (Table 1).

Cloning and sequencing. The complete RT-coding regions of five 3TC-sensitive and five 3TC-resistant (2.5 μM) variants of each of pJ5 and pC8 as well as four 3TC-sensitive and eight 3TC-resistant (750 μM) pC8 variants were amplified from the genomic DNAs of C8166-infected cells with the SRT01-SRT02 primer pair (Table 2). PCR-amplified DNA was cloned into the pCRII vector (Strategene, La Jolla, Calif.), and RT sequences were determined by double-strand sequencing (dsDNA Cycle Sequencing System; Gibco-BRL Laboratories) by using a series of nine primers, primers SRT01 to SRT09, spaced roughly 200 bp apart in the RT-coding region (Table 2).

All five of the 3TC-resistant (2.5 μM) clones contained a Met-to-Ile (ATG-to-ATA) substitution at codon 184; none of the drug-sensitive variants contained this mutation. The eight 3TC-resistant (750 μM) clones contained a Met-to-Val (ATG-to-GTA) substitution at codon 184, while the four sensitive variants contained the wild-type codon (ATG [Met]) (Table 1). Several other substitutions were observed in SIV variants selected in 750 μM 3TC. A Cys→Tyr (TGC→TAC) substitution was observed at codon 508 in four of the eight resistant clones. Codon 296 alterations were seen in two other resistant clones, i.e., Thr→Ala (ACT→GCT) or Thr→Ser (ACT→TCT). Any relevance of these latter substitutions to drug resistance will need to be assessed by site-directed mutagenesis.

Site-directed mutagenesis. To confirm the biological relevance of the M184 mutations, we introduced each of the M184I (ATA) and M184V (GTG) substitutions into the 5′ half of SIVmac239, p239SpSp5′. The SIVmac239 virus is cloned as two halves due to the instability of the full-length clone (10, 17). Toward this end, a 1.7-kb fragment (positions 4236 to 5732) of the RT-coding region, containing codon 184, was amplified by PCR and was cloned into the pCRII vector with the SRT01-SRT02 primer pair (Table 2).

Mutagenesis was performed by using the Quickchange mutagenesis kit (Strategene) with mutagenic primers 5′-CCTTA GTCCAGTAT(A/G)T(A/G)GATGACATCTTAA and 5′-GC TATTAAGATCTCATC(T/C)A(T/C)ATACTGGACTAAGG (M184I and M184V) and was confirmed by sequencing. A 754-bp *Bst*II fragment containing codon 184 was cloned back into p239SpSp5′. Mutant proviruses were regenerated by *Sph*I digestion of p239SpSp5′ and p239SpE3′ and ligation of a mixture of the two plasmids. The proviral DNA yielded virus upon transfection into COS-7 T cells and subsequent amplification in C8166 cells. SIVmac239 wild-type, M184V, and M184I virus strains were tested for their susceptibilities to a variety of antiviral compounds.

The 50% inhibitory concentrations (IC_{50}s) were determined on the basis of RT activity in culture fluids as described previously (4, 8), and the role of the M184I and M184V mutations in SIV resistance to 3TC was substantiated (Table 3). Although these IC_{50}s are higher than those seen for HIV-1, they are in agreement with previous findings for SIV (24) and feline immunodeficiency virus (21) for nucleoside analogs. The M184V substitution in SIV appeared to confer a modest increase in the IC_{50} of ddI but not in that of ddC. In contrast, none of the 3TC-resistant clones displayed resistance to AZT or to the protease inhibitors indinavir and saquinavir. The M184V and M184I substitutions also seemed to confer increased sensitivity to d4T. This needs to be further assessed. SIV, like HIV-2, is not sensitive to nonnucleoside RT inhibitors (2); in our experiments, all SIV strains tested were insensitive to nevirapine.

These studies confirm the role of the M184I and M184V substitutions in SIV RT as encoding resistance to 3TC. As in the case of HIV-1, we found that tissue culture passage first selected for M184I, which, with time, became overgrown by M184V-containing variants. As in the case of HIV-1, amino acid 184 in the SIV RT is found in the highly conserved YMDD region essential for enzyme function (13, 25). A mutation from methionine to leucine at position 184 was reported to reduce enzyme activity by more than 80% (14). In FIV, substitution of methionine to valine or threonine in the YMDD motif conferred resistance to 3TC (21).

Recent studies have shown that HIV-1 has a propensity to favor G→A substitutions in the context of 3TC pressure (11). Hence, the M184I change (ATG→ATA) is initially favored over M184V (ATG→GTG), which is independently selected by 3TC from wild-type virus due to its ability to confer higher-level resistance (11). In the case of SIV, it is interesting that the M184V substitution (GTA) is selected from the preexisting M184I variant (ATA) rather than directly from wild-type virus. We are currently comparing SIV variants that contain these position 184 substitutions with wild-type viruses with regard to viral replication efficiency and RT activity. In the case of HIV-1, the 184V mutation results in marginally diminished replication competence in primary cells but not cell lines (1, 15) as well as diminished RT processivity (1, 5).

Thus, the M184I and M184V mutations in SIV RT can encode resistance to 3TC. It will be interesting to determine whether SIV-infected macaques that are treated with 3TC also develop these same substitutions in their viral quasispecies.

TABLE 3. Confirmation of relevance of M184I and M184V substitutions regarding resistance to 3TC by site-directed mutagenesis

Virus, mutation	IC_{50} (μM)[a]							
	3TC	AZT	ddI	ddC	d4T	Nevirapine	Indinavir	Saquinavir
SIVmac239, M184 (wild type)	1.8 ± 0.6	0.37 ± 0.03	11.0 ± 0.9	1.0 ± 0.07	9.0 ± 0.87	>10	0.015 ± 0.008	0.006 ± 0.002
SIVmac239, M184V	>500	0.24 ± 0.01	20.0 ± 1.1	0.6 ± 0.01	2.0 ± 0.09	>10	0.015 ± 0.006	0.003 ± 0.005
SIVmac239, M184I	>500	0.40 ± 0.04	7.0 ± 0.4	2.0 ± 0.05	1.8 ± 0.06	>10	0.02 ± 0.001	0.0015 ± 0.007

[a] Results were calculated on the basis of RT activity in culture fluids. Data are means ± standard deviations for three replicate samples.

Elana Cherry and Mark Slater contributed equally to this work.

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