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MAJOR ARTICLE

Emergence of Minor Populations of Human Immunodeficiency Virus Type 1 Carrying the M184V and L90M Mutations in Subjects Undergoing Structured Treatment Interruptions

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The use of structured treatment interruption (STI) in human immunodeficiency virus (HIV)–infected subjects is currently being studied as an alternative therapeutic strategy for HIV-1. The potential risk for selection of drug-resistant HIV-1 variants during STI is unknown and remains a concern. Therefore, the emergence of drug resistance in sequential plasma samples obtained from 28 subjects with chronic HIV infection was studied. They underwent 4 cycles of 2-week STI, followed by 8-week retreatment with highly active antiretroviral therapy identical to that used before STI, and they had never failed treatment before undergoing STI. At week 40, treatment was stopped for a longer period. Minor populations of drug-resistant variants were detected by quantitative real-time polymerase chain reaction, by use of allele-discriminating oligonucleotides for 2 key resistance mutations: L90M (protease) and M184V (reverse transcriptase). The approximate discriminative power was 0.1%. In 14 of 25 and in 3 of 25 subjects, the M184V and the L90M mutations, respectively, were detected as minor populations, at different times during STI. Overall, these results indicate that, in subjects undergoing multiple STIs, HIV-1 variants carrying drug-resistance mutations can emerge during periods of increased HIV-1 replication.

Highly active antiretroviral therapy (HAART) has reduced the morbidity and mortality of subjects infected with human immunodeficiency virus type 1 (HIV-1) [1, 2]. However, the existence of a long-lived reservoir of latently infected $CD4^+$ T lymphocytes makes it unlikely that HIV-1 can be eradicated by currently available antiretroviral drugs [3–7]. Adverse effects and long-term toxicity, associated with prolonged use of these drugs, has become a critical issue when considering the beneficial effects of HAART [8, 9].

Structured treatment interruption (STI) is an alternative therapeutic strategy for HIV-1 that is currently under investigation. STI is used as a means of boosting HIV-1–specific immune responses, while, at the same time, reducing the adverse effects, toxicity, and cost associated with HAART. Encouraging results from the use of STI in subjects with acute HIV-1 infection, the majority of whom controlled viral replication after cessation of treatment, have been reported [10]. The clin-

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ical benefits of STI in subjects with chronic HIV-1 infection remain unclear [11–13]. The largest STI trial undertaken thus far, the Swiss-Spanish Intermittent Treatment Trial (SSITT), enrolled 133 subjects with chronic HIV-1 infection who underwent STI after successful HAART. The results of that trial did not show a relationship between control of HIV-1 viremia and induction of virus-specific cytotoxic T lymphocytes [14–16]. However, SSITT demonstrated that STI is safe in subjects with chronic HIV-1 infection. The risk of selecting drug-resistant HIV-1 variants during STI is unknown and remains a concern [17]. A recent report demonstrates selection of drug-resistant viruses in the context of STI: 2 of 12 subjects with chronic HIV-1 infection developed the M184V mutation, which is associated with reduced susceptibility to lamivudine [18].

Genotypic mutations associated with drug resistance are generally detected by direct sequencing or hybridization to allele-specific oligonucleotides. One drawback to these techniques is their inability to detect and quantify minor (<20%) populations of either the wild-type or mutant variants. This is mainly due to the failure of polymerase chain reaction (PCR) primers to discriminate, and subsequently to amplify, variants with a low molar ratio [19, 20]. In a previous study, we reported a new method for differential amplification of simian immunodeficiency virus species that is based on real-time PCR using molecular beacons and selective oligonucleotides [21]. Subsequently, other groups have reported similar assays for the selective quantification of resistant HIV-1 viral sequences [22]. In the present study, this technique was modified to detect 2 key HIV-1 drug-resistance mutations, L90M (protease) and M184V (reverse transcriptase [RT]). This assay allows simultaneous detection of both the wild-type and drug-resistant variants in the same sample, enabling direct quantification and analysis of changes in each virus population.

To evaluate the development of minor drug-resistant HIV-1 variants, we used this assay to assess subjects with chronic HIV-1 infection during STI. Our results indicate the presence of minor populations of HIV-1 with L90M and M184V mutations in subjects undergoing STI.

SUBJECTS, MATERIALS, AND METHODS

Study design and subjects. Of the 29 subjects enrolled from the Zurich cohort of the SSITT [14], 28 participated in the present study. Of those, 15 participated in an extended protocol with frequent blood sampling [16]. All subjects also participated in the Swiss HIV Cohort Study.

To be eligible for the SSITT, subjects had to be receiving their first combination antiretroviral therapy (ART) with 2 or 3 drugs (excluding nonnucleoside RT inhibitors) for >6 months and never have experienced therapy failure. Subjects who had received previous nonsuppressive treatment with 1 or 2 nucleoside RT inhibitors were excluded. Plasma viremia had to be <50 HIV-1 RNA copies/mL for at least 6 months, and $CD4^+$ T cell counts had to be >300 cells/mL. The baseline characteristics of the study subjects are summarized in table 1. The trial consisted of 4 cycles, each consisting of a 2-week treatment interruption followed by resumed treatment for 8–10 weeks. Subjects whose plasma viremia did not decrease to values of <50 HIV-1 RNA copies/mL of plasma during periods of resumed treatment were excluded from the study. After 4 STI cycles (week 40), treatment was stopped for at least 3 months, unless symptoms of acute HIV-1 infection occurred, $CD4^+$ T cell counts decreased to <300 cells/mL, or virus load exceeded the following predetermined values: 3 consecutive measurements of >50,000, 2 of >100,000, or 1 of >500,000 HIV-1 RNA copies/mL of plasma. At week 52, treatment was resumed as described elsewhere [14, 16]. The SSITT was approved by the Ethics Committee of the University Hospital Zurich, and written, informed consent was obtained from each subject.

Blood sampling and HIV-1 quantification. Blood samples were obtained on days 0, 4, 8, 14, 18, 22, 24, 28, 35, 42, 56, 63, and 70 of each cycle, for the extended protocol, and on days 0, 14, and 63 of each cycle, for the basic SSITT protocol. From week 40 to 52, blood was drawn weekly from all subjects. Plasma HIV-1 RNA was quantified by use of an Amplicor HIV-1 Monitor test (version 1.5; Roche Diagnostics) with ultrasensitive modifications, resulting in a level of detection of $\leqslant 50$ HIV-1 RNA copies/mL of plasma [23, 24].

HIV-1 RNA from subjects' plasma. Plasma samples collected from blood in EDTA anticoagulant were centrifuged at 2000 *g* for 5 min, to pellet cell debris. Particle-associated HIV-1 RNA was purified from cell-free plasma (250–500 μL) by use of a QIAamp Viral RNA Mini Kit (Qiagen), in accordance with the manufacturer's instructions for large sample volumes. Part of each RNA sample was used for cDNA synthesis immediately after extraction, and the remainder was stored at $-80°C$.

Construction of DNA and RNA standards for quantitative real-time PCR for differential amplification of L90M and M184V mutations. The primers for PCR and RT-PCR were designed on the basis of published sequences within the *pol* region of HIV-1_{HXB2}. Oligonucleotides were synthesized by Operon Technologies (Alameda, California). A plasmid encoding HIV-1_{HXB2} (Aaron Diamond AIDS Research Repository) was used as a background DNA template for the construction of DNA and RNA transcripts. Regions within the *pol* gene of HIV-1_{HXB2} were cloned into pGEM-T (Promega), in accordance with the manufacturer's instructions. The L90M (TTG to ATG transversion in codon 90 of the protease gene) and M184V (ATG to GTG transversion in codon 184 of the RT gene) drug-resistance mutations were introduced into the pGEM-T HIV-1_{HXB2} plasmid by site-directed mutagenesis, by use of a QuikChange Site-Directed Mutagenesis Kit (Stratagene) and the

Table 1. Baseline characteristics of subjects before structured treatment interruptions (STIs).

Subject	Age, years	Sex	Route of infection	Duration of HIV-1 infection, years	CD4 cell counts, cells/mm^3	HIV subtype	VL before HAART, copies/mL	HAART received before STI[b]	Time of VL <50 copies/mL before STI, months
101	40	M	Het	>14	719	B	46,452[a]	ddI, d4T, NFV	17
102	41	M	MSM	>7	723	B	561,831	AZT, 3TC, IDV	32
103	42	M	Het	>7	272	E/CRF1	26,568[a]	ddI, d4T, NFV	12
104	47	M	MSM	>2	526	A	1,502,850	AZT, 3TC, NFV	26
105	52	F	Het	3	1269	B	128,555[a]	AZT, 3TC, IDV	26
106	39	M	IVDA	>9	878	B	506[a]	AZT, 3TC	36
107	44	F	Het	>7	544	B	5216	AZT, 3TC, NFV	25
109	38	M	MSM	>9	1115	B	34,752	AZT, 3TC, RTV	31
111	38	M	MSM	>3	422	B	122,902[a]	ddI, d4T, NFV	11
112	46	M	IVDA	>4	347	B	32,608[a]	AZT, 3TC, IDV	25
113	59	M	MSM	>3	995	B	107,303	AZT, 3TC, RTV	20
114	32	M	MSM	>7	907	B	9275	AZT, 3TC, RTV	29
115	24	M	MSM	>2	570	B	54,693[a]	d4T, 3TC, NFV	21
116	53	M	Het	>2	350	B	467,593	AZT, 3TC, IDV	32
117	33	F	IVDA	4	489	B	29,345[a]	AZT, 3TC, RTV	36
118	33	M	MSM	4	832	B	16,927	AZT, 3TC, IDV	30
119	35	M	Het	>1	440	B	114,117[a]	d4T, 3TC, SQV, RTV	12
120	55	M	Het	>2	766	B	162,701[a]	AZT, 3TC, IDV	28
121	38	M	Het	>2	591	B	164,772	d4T, 3TC, NFV	12
122	40	M	MSM	>14	669	B	13,348[a]	AZT, 3TC, IDV	30
123	39	F	IVDA	>8	1335	B	14,410	AZT, 3TC, RTV	25
124	43	M	IVDA	>2	715	B	19,453[a]	ddI, d4T, NFV	19
125	35	F	Het	>5	777	E/CRF1	11,298	ddI, d4T, NFV	23
126	49	M	MSM	>7	842	B	63,698	AZT, 3TC, RTV	34
127	52	F	Het	>4	839	B	25,417	d4T, 3TC, NFV	22
128	43	F	IVDA	>2	749	B	9404	AZT, ddI, NFV	25
129	44	F	Het	>1	639	C	1,287,812	AZT, 3TC, IDV	29
130	66	M	Het	1	670	A	821[a]	AZT, 3TC, NFV	30
Median	42			4	717		33,680		26
Average	43			5	720		179,808		25
SD	9			4	261		368,727		7

NOTE. 3TC, lamivudine; AZT, zidovudine; d4T, stavudine; ddI, didanosine; HAART, highly active antiretroviral therapy; Het, heterosexual; HIV, human immunodeficiency virus; IDV, indinavir; IVDA, intravenous drug abuse; MSM, men who have sex with men; NFV, nelfinavir; RTV, ritonavir; SQV, saquinavir; VL, virus load.

[a] Mean pretreatment HIV-RNA values when 2 values before initiation of HAART were available.

[b] All subjects reached full suppression of plasma viremia (<50 copies/mL) after receiving these combination antiretroviral drug regimens and had never received nonsuppressive mono- or double nucleoside reverse-transcriptase inhibitor therapy.

oligonucleotides tgL90M 5′-CTGTCAACATAATTGGAAGA-AATCTGATGACTCAGATTGGTTGCAC-3′ (nt 2494–2539), tgL90Mrc 5′-GTGCAACCAATCTGAGTCATCAGATTTCTTC-CAATTATGTTGACAG-3′ (nt 2494–2539), tgM184V 5′-GACA-TAGTTATCTATCAATACGTGGATGATTTGTATGTAGGATC-TGAC-3′ (nt 3078–3125), and tgM184Vrc 5′-GTCAGATCCTAC-ATACAAATCATCCACGTATTGATAGATAACTATGTC-3′ (nt 3078–3125), in accordance with the manufacturer's instructions. The introduction of each mutation into the pGEM-T HIV-1_{HXB2} template DNA was confirmed by sequencing.

DNA standards for quantification were prepared by PCR from plasmid DNA constructed by in vitro mutagenesis. Wild-type and mutant L90M-M184V plasmid DNA were amplified by PCR, and the amplicons were purified from excess primers, dNTPs, and protein by use of a QIAquick PCR purification Kit (QIAGEN). The PCR contained 1× PCR buffer (QIAGEN), 0.5 mmol/L dNTPs (Gibco BRL), 0.4 μmol/L each primer (pol 2259 5′-GTCACTCTTTGCCAACGACC-3′ [HXB2 2259–2279] and pol 3287 5′-CAGCACTATAGGCTGTACTGTC-3′ [HXB2 3266–3287]) and 2.5 U of HotStar*Taq* DNA polymerase (QIAGEN; final volume, 50 μL). The thermal-cycling conditions consisted of 15 min at 95°C, to activate the DNA polymerase, followed by 40 cycles (30 s at 94°C, 30 s at 55°C, and 1 min at 72°C) and a termination of 5 min at 72°C. The con-

centrations of the purified wild-type and L90M-M184V amplicons (dsDNA, 1029 nt in length) were measured by UV-absorbance spectrophotometry. Ten-fold serial dilutions of the purified dsDNA transcripts with known molar concentrations were used as templates to generate the standard curves used for selective amplification by fluorescence-based real-time PCR.

RNA transcripts corresponding to the wild-type and L90M and M184V drug-resistance mutations were prepared by in vitro transcription of PCR products that contained the T7 RNA polymerase promoter site, by use of the MEGAscript T7 kit (Ambion). DNA templates for in vitro transcription were generated by PCR from wild-type and L90M-M184V plasmid DNA. The PCR contained 1× PCR buffer, 0.5 mmol/L dNTPs, 0.4 μmol/L each primer (T7-pol 2258 5′-TAATACGACTCACTATAGGGGTCACTCTTTGCCAACGACC-3 [nt 2258–2279; T7 promoter sequence is underlined] and pol 3458 5′-TTAGAATCTCTCTGTTTTCTGCC-3′ [HXB2 3458–3480]) and 2.5 U of HotStar*Taq* DNA polymerase (final volume, 50 μL). The thermal-cycling conditions consisted of 15 min at 95°C, followed by 40 cycles (30 s at 94°C, 30 s at 55°C, and 80 s at 72°C) and a termination of 5 min at 72°C. The concentrations of the purified DNAse-treated RNA transcripts (1223 nt in length) were measured by UV-absorbance spectrophotometry.

Selective quantification of wild-type and L90M and M184V drug-resistant HIV-1 strains. Reverse transcription with viral RNA from plasma samples was performed as described elsewhere [21]. In brief, 20 μL of viral RNA and 0.5 μmol/L of a gene-specific primer recognizing either the HIV-1 protease gene (5′-GCCATCCATTCC-3′ [nt 2592–2603]) or the HIV-1 RT gene (5′-GGTTCTTTCTGATG-3′ [nt 3210–3223]) were used.

Amplification of HIV-1 cDNA or genomic DNA was performed by use of 2 external primer pairs in the same reaction: 1 primer pair for each region of interest (either wild type or mutant), to optimize the primer binding sites. PCRs consisted of 20 μL of cDNA, 1× PCR buffer II (Applied Biosystems), 3.5 mmol/L $MgCl_2$, 0.5 mmol/L dNTPs (GibcoBRL), 0.4 μmol/L each upstream primer (L90 EP1 5′-GAAGCTCTATTAGATACAGG-3′ [nt 2313–2332] and M184 EP1 5′-AATCCAGACATAGTTATCTATC-3′ [nt 3072–3093]), 0.4 μmol/L each downstream primer (L90 EP2 5′-TTTAAAGTGCAACCAATCTGAG-3′ [nt 2524–2545] and M184 EP2 5′-TTTTTTGTCTGGTGTGGTAAATC-3′ [nt 3187–3209]), and 2.5 U of Ampli*Taq* Gold DNA polymerase (Applied Biosystems; final volume, 50 μL). Amplification was performed for 15 cycles (15 s at 94°C, 1 min at 43°C, and 30 s at 72°C) in a Mastercycler gradient (Eppendorf Scientific). PCR products were purified by use of a QIAquick PCR purification kit (Qiagen), in accordance with the manufacturer's instructions.

Nested real-time amplification of HIV-1 DNA was performed separately for each region of interest (either wild type or mutant), by use of 10 μL of first-round PCR product, 1× x-rhodamine–PCR buffer, 3.5 mmol/L $MgCl_2$, 0.5 mmol/L dNTPs (GibcoBRL), 0.2× SYBR green (Molecular Probes), 0.4 μmol/L each primer (pol 2316 5′-GCTCTATTAGATACAGGAGCAG-3′ [nt 2316–2337] and IN L90 5′-TGCAACCAATCTGAGTCIA-3′ [nt 2520 -2538] or IN L90M 5′-TGCAACCAATCTGAGTCIT-3′ [nt 2520–2538]; and pol 3206 5′-TTTGTCTGGTGTGGTAAATCCCCAC-3′ [nt 3182–3206] and IN M184 5′-CCAGACATAGTTATCTATCAATA IA-3′ [nt 3075–3099] or IN M184V 5′-CCAGACATAGTTATCTATCAAT AIG-3′ [nt 3075–3099]), and 2.5 U of Ampli*Taq* Gold DNA polymerase (Applied Biosystems; final volume, 50 μL). Fifty cycles of amplification (15 s at 94°C, 30 s at 60°C, and 30 s at 72°C) were performed in an Applied Biosystems 7700 Prism spectrofluorometric thermal cycler (Applied Biosystems).

DNA standards were tested in duplicate for each experiment. The standards were prepared by serial dilution from 10^6–10 copies/reaction. Viral RNA samples were also tested in duplicate. Copy numbers were calculated by interpolation of the experimentally determined threshold cycle for the test specimen onto a control standard regression curve [25]. The ratio of wild-type and mutant sequences was calculated on the basis of copy numbers for each population. Nested real-time PCR assays have a detection limit of 10 HIV-1 DNA copies/reaction, with a linear dynamic range of >6 logs.

Since low virus loads diminish the discriminatory ability of each assay, the final calculation of the percentage of minor variants was dependent on the virus load. On the basis of the protocol used in the present study, the equivalent of 11.2% of the virus load (in RNA copies per milliliter) was estimated for use in the first round of PCR and, subsequently, in the quantitative real-time PCR assays for differential amplification (virus load in 1 mL of plasma ≅ 100%; 560 μL of plasma used for RNA isolation ≅ 56%; two-fifths of eluted RNA used for cDNA synthesis ≅ 22.4%, and half of cDNA used for first round of PCR ≅ 11.2%). For each sample, the limit of detection of minority variants was calculated on the basis of the virus load. For instance, a virus load of 893 RNA copies/mL of plasma results in 100 cDNA copies (11.2%) used in the first round of amplification. Therefore, the equivalent of 100 cDNA copies is used in quantitative real-time PCR for differential amplification. Because of the detection limit of 10 DNA copies/reaction in each real-time PCR, at least 10 copies of the initial 100 cDNA copies have to carry the mutant to be detectable; this equals 10% in this example—anything <10% is not detectable. Thus, an individual cut-off value was estimated for every sample. Therefore, samples with virus loads of <500 HIV-1 RNA copies/mL of plasma were not tested. The results of quantitative real-time PCR assays were then compared with these calculations and were adjusted such that detection of minor populations was considered to be negative when the limit of detectable minorities was higher on the basis of individual cut-off values.

RESULTS

Differential amplification of HIV-1 variants by quantitative real-time PCR. The quantitative real-time PCR assay used in the present study is based on the amplification refractory mutation system (ARMS) [26]. This assay uses allele-specific oligonucleotides for detection of either wild-type or mutant sequences (figure 1*A*). To evaluate the discriminatory ability of each assay, wild-type and mutant DNA standards were used for amplification with the corresponding and the noncorresponding oligonucleotides. Amplification of L90M DNA by the wild-type–specific primer was detected by a positive fluorescent signal, which appeared ~14 PCR cycles after the signal for the wild-type target. This is equivalent to a decreased efficiency of amplification of >10,000-fold (figure 1*B*). The discriminatory ability (ΔC_T) for the amplification of both targets, with the mutation-specific primer, is 16, which is equivalent to a >60,000-fold decrease in efficiency of amplification of the incorrect target. Evaluating the discriminatory ability of the M184/V assay resulted in ΔC_T values of 11 and 10 for M184 and M184V, respectively (data not shown).

In addition, the discriminatory ability of each assay was tested in reciprocal mixing experiments by adding 10^6 copies of noncomplementary DNA to a serial dilution (10^6–10 copies) of either wild-type or mutant standard DNA. In each case, the threshold cycle was compared to PCRs performed without the addition of noncomplementary DNA. The discriminatory ability of this assay was comparable to that obtained from the experiments described above (figure 1*C*). Taking into account SD, the estimated discriminatory abilities for the different wild-type and mutant sequences are as follows: 0.01% for detection of L90 wild type as minority population, 0.01% for L90M, 0.1% for M184, and 0.2% for M184V. The dynamic range of all assays is >6 logs.

To overcome the problem of HIV-1 heterogeneity, multiplex PCR with external primer pairs was performed before the samples were amplified by quantitative real-time PCR. Oligonucleotides were chosen, covering almost the entire primer binding site used for selective amplification. Only a few PCR cycles at low annealing temperatures were necessary to prepare viral genomes from different subjects, for quantitative real-time PCR. Standard DNA was tested by use of 1 or all external primer pairs in the same reaction. No differences in threshold cycles and discriminatory abilities were observed. Clones with different known mutations in primer binding sites were first amplified with external primer pairs and then were used for nested quantitative real-time PCR for differential amplification. Use of sequences carrying 1 or 2 mutations in primer binding sites did not result in any significant difference in threshold cycles and discriminatory ability, compared with standard templates that were completely homologous. When sequences contained 3 or 4 mutations in the primer binding site, a positive fluo-

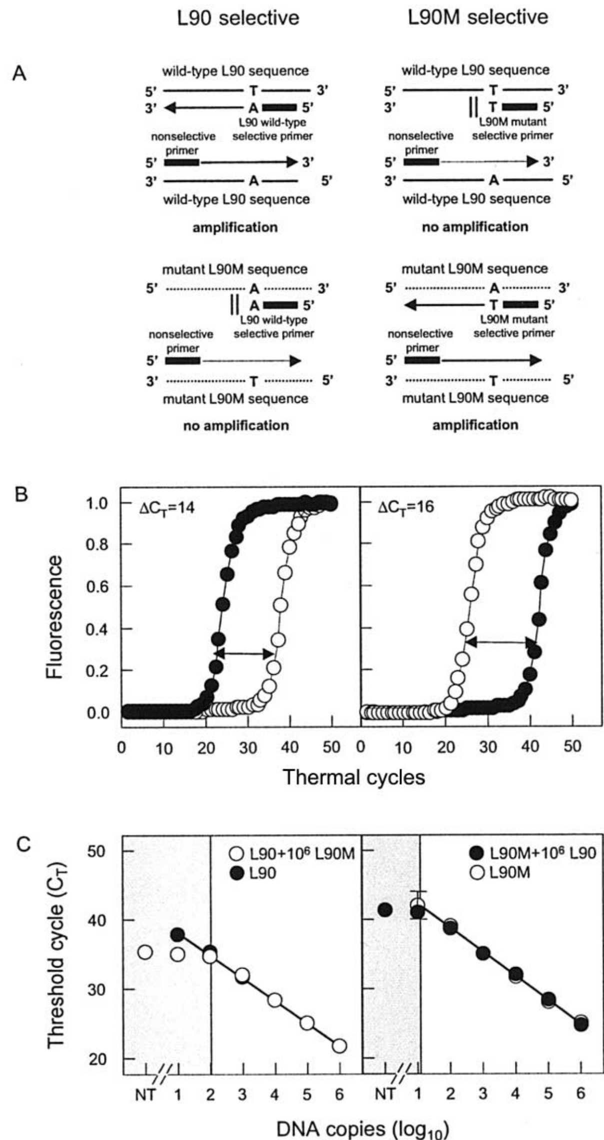

Figure 1. Scheme and discriminatory ability of quantitative real-time polymerase chain reaction (PCR) for differential amplification. *A,* Wild-type– or mutant-specific primers and a nonselective primer were used to amplify the region of interest. Amplicons were quantified by real-time PCR by use of SYBR green. *B,* Equal amounts of either wild-type (●) or mutant (○) standard DNA templates were amplified with the wild-type–specific *(left panels)* or mutation-specific *(right panels)* primers. The discriminatory abilities (ΔC_T) are indicated in the upper left corner, for each assay. *C,* Discriminatory ability of real-time PCR for differential amplification. Amplification of wild-type human immunodeficiency virus type 1 (HIV-1) DNA was performed by use of a serial dilution of standard wild-type HIV-1 DNA either with (○) or without (●) the addition of 10^6 copies of standard mutant HIV-1 DNA. Reactions were performed in duplicate, and the mean values are shown. The solid lines indicate amplification without the addition of mutant DNA *(left panel).* Amplification of mutant HIV-1 DNA was performed by use of a serial dilution of standard mutant HIV-1 DNA either with (●) or without (○) the addition of 10^6 copies of standard wild-type HIV-1 DNA. The linear standard curve for amplification of mutant DNA alone is shown in black *(right panel).* SDs are contained within the data points. NT, without correct DNA template.

rescent signal appeared >4 cycles later. However, the discriminatory ability was not affected (data not shown).

Quantification of minor populations of drug-resistant HIV-1 in subjects undergoing STI. A total of 28 subjects undergoing STI were enrolled in this study. The baseline characteristics of the study subjects are shown in table 1. All subjects had chronic HIV-1 infection and were successfully treated with HAART (virus load of <50 HIV-1 RNA copies/mL of plasma). At least 1 protease inhibitor was included in the treatment regimens of 27 subjects. All subjects were evaluated for HIV-1 carrying the L90M and M184V mutations. Subjects 102, 103, 109, and 116 had to be retreated, according to the protocol, between weeks 40 and 52. Subject 104 dropped out of the study because his virus load measurements were >50 HIV-1 RNA copies/mL of plasma after 10 weeks of retreatment. Analyses of viral and immunological responses in these subjects have been published elsewhere [14–16, 27].

The assay for detection of the L90M mutation was performed on samples from 27 subjects. It was not possible to successfully amplify wild-type or mutant sequences from 2 of these subjects (129 and 130). These subjects were infected with HIV-1 subtypes A and C, respectively. The L90M mutation was detected as a minor variant in 3 of 25 subjects (subjects 102, 116, and 118) (table 2). In subject 102, 5.6% of the total HIV-1 population carried the L90M mutation during the first cycle of STI (figure 2). Interestingly, the L90M mutation was not detected in this subject's virus population during subsequent STI cycles. In subject 116, 0.3% of the virus population carried the L90M mutation during the third cycle of STI. Similarly, in subject 118, 0.06% of the virus population carried the L90M mutation at 1 time point during the fifth cycle of STI. No evidence was found for the presence of the L90M mutation in viruses from any other study subjects.

All 28 subjects were tested for the presence of the M184V mutation. We were unable to amplify either wild-type or M184V mutant sequences in samples from 3 subjects (103, 129, and 130). These subjects were infected with HIV-1 subtypes different from subtype B (table 1). Overall, drug-resistant HIV-1 variants carrying the M184V mutation were detected in 14 of 25 subjects. In 9 subjects, minority virus populations carrying the M184V mutation were detected at only 1 or 2 time points. In 7 subjects, this mutation was detected once, as a minority, during the fifth cycle of STI (see "subject 106" in figure 2 and table 2), with percentages ranging from 0.3% to 9.8% (average, 3.1%). The M184V mutation was not detected in subsequent samples from these subjects. Among viral sequences identified for subject 127, 5.4% carried the M184V mutation during the second STI cycle, but not during subsequent STI cycles. In subject 106, the M184V mutation represented 56.9% of the sequences detected during the beginning of the fifth STI cycle. This population decreased to 1% 2 weeks later and decreased to <0.3% after an additional 2 weeks. Lower virus loads in subsequent samples did not allow us to discriminate between wild-type and mutant variants below the range of 2.6%–6.6%. Because of low virus loads during the first 4 STI cycles, only time points from the fifth STI cycle were measured. However, calculating the positive values of the M184V mutation relative to the virus load revealed that 56.9% corresponded to ~782 HIV-1 RNA copies/mL of plasma. Because of a rapid increase in virus load in this subject (106), 1% corresponds to ~451 HIV-1 RNA copies/mL of plasma. This indicates that the wild-type population was increasing during this period, whereas the M184V mutant population was slowly decreasing.

Five subjects showed diverse patterns, with regard to the appearance of the M184V mutation (figure 2 and table 2). Analysis of samples from subject 102 revealed a continuous decrease in viruses carrying the M184V mutation, during the first 3 cycles of STI (20.1%, 5.2%, and 4.9%, respectively). The M184V mutation was not detected within the range of detectable minor variants during subsequent STI cycles. This subject resumed ART (zidovudine, lamivudine, and indinavir) on day 329. The majority of viruses identified for subject 104 carried the M184V mutation at baseline, with 93.9% detected by day 8 of the first STI cycle. After reintroduction of HAART, the virus load decreased; however, the M184V mutation was detectable at levels of ~60%–80%, up to day 70. This subject subsequently dropped out of the STI study because his virus load did not decrease to <50 HIV-1 RNA copies/mL of plasma after the first STI cycle. Viremia continued to decrease but remained detectable at low levels (<500 HIV-1 RNA copies/mL of plasma). Five months later, virus load rebounded to >1000 HIV-1 RNA copies/mL of plasma. Population sequencing revealed the M184V mutation and the protease inhibitor–associated mutations 46L, 54V, 63P, and 82A. A salvage regimen was started with abacavir, saquinavir, ritonavir (changed to lopinavir/ritonavir after 20 months), and stavudine. Efavirenz was added to the regimen after 1 month. Virus load decreased and remained undetectable in plasma for the duration of follow-up (figure 2).

Viral variants carrying the M184V mutation were detectable as a minority population in samples obtained from subject 105 during the fifth STI cycle. The percentage of M184V mutants varied between 0.5% and 11% during this time but did not disappear, despite the fact that the subject was not receiving ART. Time points before the fifth STI cycle were not analyzed, because of low virus load. The first sample from subject 112 that was measured was obtained during the third STI cycle; samples of earlier STI cycles have not been used for differential amplification, because of low virus loads. Approximately 50% of the virus population from subject 112 carried the M184V mutation during the third STI cycle. During the fourth STI cycle, the M184V mutation was not detectable (<2.1%), but it

Table 2. Detection of key resistance mutations L90M and M184V in subjects undergoing structured treatment interruptions (STIs).

Subject	Drug-resistant mutation	STI cycle: First — Drug-resistant mutation, mean ± SD, %	First — Positive samples/ samples tested	Second — Drug-resistant mutation, mean ± SD, %	Second — Positive samples/ samples tested	Third — Drug-resistant mutation, mean ± SD, %	Third — Positive samples/ samples tested	Fourth — Drug-resistant mutation, mean ± SD, %	Fourth — Positive samples/ samples tested	Fifth — Drug-resistant mutation, mean ± SD, %	Fifth — Positive samples/ samples tested
102	M184V	20.1 ± 1.0	1/2	5.2 ± 1.5	1/3	4.9 ± 0.2	1/3	…	0/1	…	0/5
	L90M	5.6 ± 0.3	1/1	…	0/3	…	0/3	…	0/1	…	0/3
104	M184V	59.3–93.9 ± 0.8–34.0	6/7	Drop out							
105	M184V	NA		NA		NA		NA		0.5–11.0 ± 0.0–0.4	9/12
106	M184V	NA		NA		NA		NA		59.9 ± 3.1	2/11
										1.0 ± 0.3	
112	M184V	NA		NA		49.7 ± 1.0	1/1	…	0/1	1.7–46.4 ± 3.0–1.6	6/7
113	M184V	NA		NA		NA		NA		5.2 ± 0.1	1/11
115	M184V	NA		…	0/1	NA		NA		0.9 ± 0.8	1/6
116	M184V	0.9 ± 0.1	1/4	0.3 ± 0.1	1/7	5.9 ± 1.1	2/2	27.3 ± 0.6	1/2	NA	
						0.8 ± 0.1					
	L90M	…	0/2	…	0/3	0.3 ± 0.0	1/3	…	0/1	NA	
118	L90M	…	0/1	…	0/1	…	0/1	…	0/1	0.06 ± 0.05	1/4
120	M184V	NA		…	0/1	NA		NA		0.3 ± 0.0	1/10
121	M184V	…	0/2	…	0/1	…	0/1	…	0/1	0.4 ± 0.1	1/8
122	M184V	…	0/1	…	0/1	…	0/1	…	0/1	9.8 ± 0.7	1/3
124	M184V	NA		NA		NA		NA		2.9 ± 0.3	1/4
125	M184V	NA		NA		NA		NA		2.6 ± 0.5	1/4
127	M184V	…	0/2	5.4 ± 1.5	1/2	…	0/2	…	0/1	NA	
Total	M184V	3		3		3		1		10	
Total	L90M	1		0		1		0		1	

NOTE. The SD of the estimates of percentage of mutant virus was derived on the basis of the δ method. NA, not applicable, due to either very low virus load, negative results for both wild-type and mutant sequences, or no sample availability.

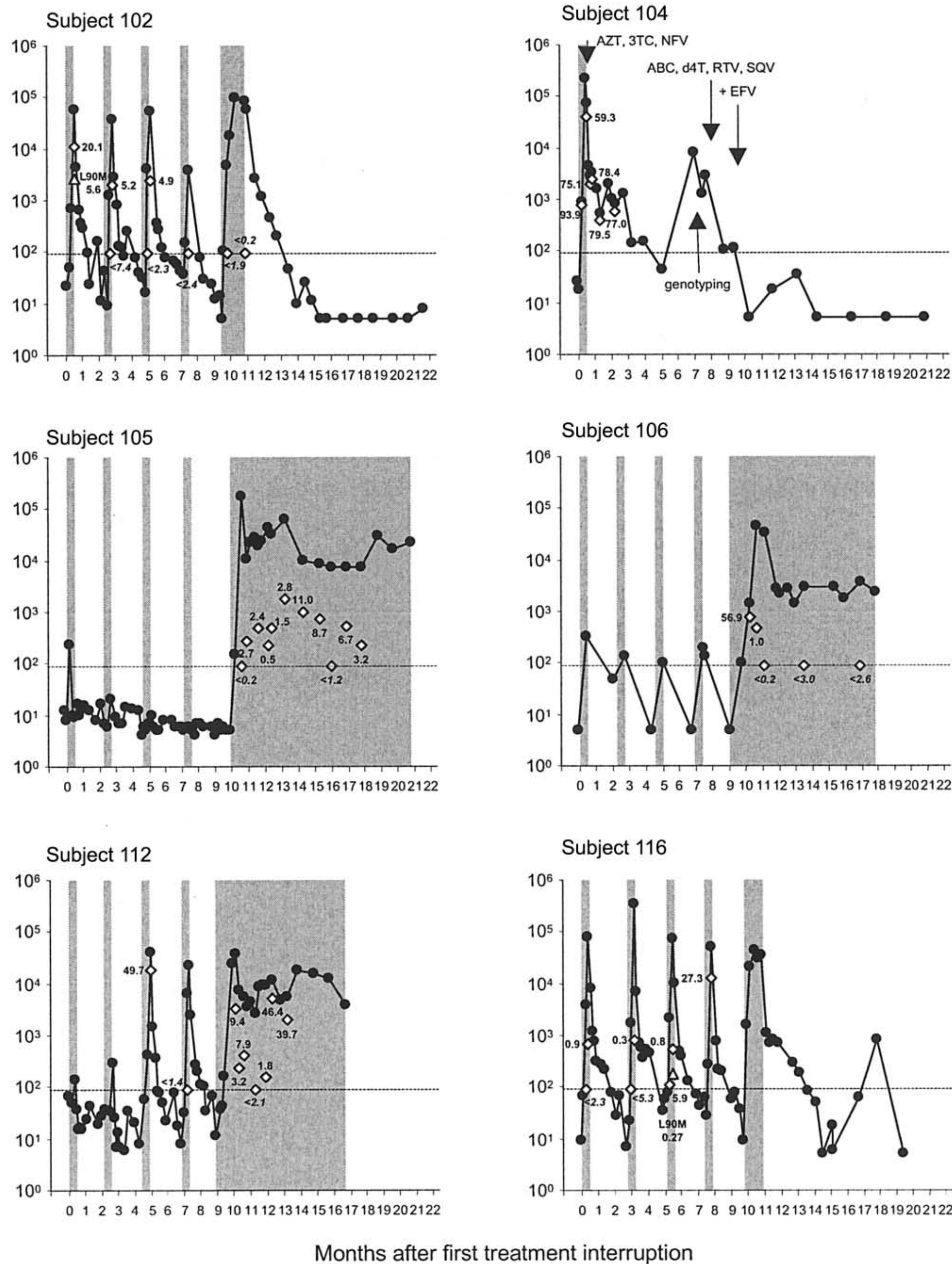

Figure 2. Kinetics of virus load and detection of drug-resistant viral sequences in subjects undergoing structured treatment interruption (STI). Human immunodeficiency virus type 1 (HIV-1) RNA in plasma was measured by use of an Amplicor HIV-1 Monitor test (version 1.5; Roche Diagnostics) (●). Drug-resistant variants carrying either the L90M or M184V mutations were detected by quantitative real-time polymerase chain reaction for differential amplification. The percentage of the virus population carrying the specific mutation was used to calculate the absolute HIV-1 RNA copies per milliliter of plasma, on the basis of the corresponding virus load measurement. Copy numbers representing the L90M variants (△) and M184V variants (◇) are shown. Numbers represent percentages of each drug-resistant virus population. Percentage values below the limit of detectable minorities, because of low virus load, are printed in italic type. The limit of sensitivity (90 copies of HIV-1 RNA) is indicated by the dotted line. The shaded areas represent periods of STI comprising 4 cycles; each cycle comprised 2 weeks without HAART followed by 8 weeks of resumed treatment. Treatment was discontinued for at least 3 months, at week 40, and was resumed if necessary. Subject 104 dropped out of the study because his virus load did not decrease to <50 HIV-1 RNA copies/mL of plasma after the first STI cycle.

appeared again during the fifth STI cycle, showing an increase over time. In subject 116, the M184V mutation was detectable as a minor variant during the first STI cycle (0.9%), the second STI cycle (0.3%), and the third STI cycle, at days 148 (5.9%) and 154 (0.8%). During the fourth STI cycle, the level of viruses carrying the M184V mutation reached 27.3%. HAART was reintroduced in this subject 33 days after the fifth STI.

All subjects had received their first ART before they were enrolled in SSITT. To determine whether minor populations carrying the L90M and M184V mutations were already present, before initiation of HAART, in our subjects' virus populations, we also tested plasma samples obtained from 24 of these subjects when they were still drug naive—that is, before any ART was started. No successful amplification of wild-type or mutant sequences was possible in samples from 3 (L90/M) and 2 (M184/V) subjects, respectively. In the remaining samples, no minor variants of viruses carrying the L90M or M184V mutations were detectable. With regard to the L90M mutation, frequencies were <0.03%–<1% (median, <0.2%) in samples from 21 subjects. The M184V mutation was not detectable in 22 subjects, at frequencies of <0.2%–9.1% (median, <0.5%).

DISCUSSION

The present study was undertaken to analyze the potential emergence of drug-resistant HIV-1 in subjects undergoing STI. Genotypic analysis of minor populations was performed by use of a novel, quantitative real-time PCR assay for differential amplification with selective oligonucleotides that detect 2 key resistance mutations: L90M (protease) and M184V (RT). This methodology is based on the ARMS [26]. Gene-specific oligonucleotides contained a deoxyinosine at the −2 position of the 3′ end that, because of destabilizing effects on the formation of duplexes, increased the discriminatory ability 5–10-fold (data not shown). In addition, since deoxyinosine is less tolerant of mismatches, variability in the second position, among different viral sequences, is less critical [28]. This methodology is able to detect a low percentage of minor populations of L90 wild-type and L90M drug-resistant variants (0.01%), as well as M184 wild-type (0.1%) and M184V drug-resistant variants (0.2%).

A total of 28 subjects were enrolled in the present study; 181 plasma samples from 25 subjects were analyzed for the presence of the L90M mutation. This mutation was detected as a minor population in 3 samples from 3 of 25 subjects. A total of 216 plasma samples from 25 subjects were analyzed for the presence of the M184V mutation: 40 samples from 14 subjects were positive for the M184V mutation. The predominance of the M184V mutation, compared with the L90M mutation, was not unexpected. The selection of mutations associated with drug resistance to protease inhibitors is a slow, stepwise process [29, 30], compared with the emergence of M184V, which is a single-point mutation selected by lamivudine [31]. Thus, the more frequent detection of the M184V mutation, compared with the L90M mutation, might represent a very early event in the emergence of drug resistance in our subjects undergoing several periods of significant HIV-1 replication [26].

The majority of subjects in this study were infected with HIV-1 subtype B (table 1), and samples from each of these subjects have been successfully amplified. Five subjects were infected with other HIV-1 subtypes. Although HIV-1 clones containing mutations at different positions in the primer binding sites were successfully amplified in the set up of validation of this method, only HIV-1 from 2 of these subjects was amplified by use of both L90M and M184V real-time PCR; however, it was not possible to amplify samples from 2 other subjects (129 and 130). Samples from subject 103 were successfully analyzed, with respect to the L90M mutation, but we were unable to generate amplicons to detect the M184V mutation. It is likely that the oligonucleotides did not hybridize to the HIV-1 targets of the subjects, because of too many mutations in the primer binding site. The use of external primer pairs in combination with low annealing temperatures during the first PCR reaction and a deoxyinosine at the −2 position of the 3′ end of the selective oligonucleotides are efforts to minimize the influence of differences in viral sequences. This procedure is successful, with respect to HIV-1 subtype B. However, a small risk of underestimating 1 or the other HIV-1 variant cannot completely be denied.

Sporadic occurrence of the L90M mutation was detected in 3 subjects during STI. This mutation appeared only once in each subject, during different STI cycles. The percentage of viruses carrying the L90M mutation was <0.3% in subjects 116 and 118. We cannot rule out the possibility that a minor population of L90M variants within this range may be present at other time points; however, for most of the other samples, low virus loads did not allow us to discriminate <1%. In 9 subjects, the M184V mutation was detected during the fifth STI cycle in only 1 or 2 samples. It is possible that M184V was also selected during prior STI cycles, but, because of lower virus loads during these periods, the M184V mutation was not detectable. On the other hand, more samples were suitable for testing during the last (fifth) STI cycle, increasing the probability of detecting mutations, because of the occurrence of a full rebound in virus load, compared with the shorter, prior STI cycles. Interestingly, 2 of these subjects were treatment naive with respect to lamivudine. Instead, their regimen contained didanosine. That virus strains harboring the M184V mutation show reduced sensitivity against didanosine in vitro may potentially lead to selection of minor populations carrying the M184V mutation in subjects receiving didanosine treatment [32].

Baseline samples (from the first STI cycle, if available) from 3 subjects were positive for the M184V mutation. This mutation

was not detected in subsequent samples obtained from subject 102 during successive STI cycles. In subject 116, the frequency of variants carrying M184V increased in subsequent STI cycles. Drug-specific pharmacokinetic factors were excluded as an explanation for the divergent outcomes, because subjects 102 and 116 each received the same drugs. However, we cannot rule out the possibility that other pharmacokinetic factors, such as differences in drug absorption, led to concentrations that favored the outgrowth of the M184V mutation in this subject. Of the 25 subjects studied, only subject 104 developed drug-resistance mutations associated with treatment failure. On day 8 of the first STI cycle, the majority (>93%) of viruses from this subject already harbored the M184V mutation (figure 2). This finding was confirmed by direct sequencing (L. Perrin, personal communication). The M184V mutation was found, in addition to multiple mutations associated with resistance to protease inhibitors. The subject dropped out of the study with a virus load of >50 HIV-1 RNA copies/mL of plasma after the first STI cycle. A salvage regimen was subsequently introduced with successful suppression of plasma viremia to <50 HIV-1 RNA copies/mL for >27 months.

In subject 105, minor populations carrying the M184V mutation were detected at frequencies of 0.5%–11% throughout the fifth STI cycle. No consistent pattern of change was observed, suggesting that other mutations, in addition to M184V, may influence the replication of minor variants in this subject and in others (e.g., subject 112). During the third STI cycle, approximately half of the virus population contained the M184V mutation. This mutation decreased during the fourth and fifth STI cycles, to percentages between <1.4% and 9.4%, and increased again, to 46.4%, between weeks 9 and 10 of the fifth STI cycle. Fluctuations in the frequency of the M184V mutation have been observed in some subjects. The appearance of different quasi species at different time points might reflect the heterogeneity of HIV-1. Changes in the ratio between wild-type and mutant virus populations may be related to different advantages, such as viral fitness, for certain virus populations at different stages.

There is one potential caveat to our study: the very high sensitivity of our assay to detect the 2 mutations at very low frequencies, in theory, may have lead to detection of randomly occurring minor populations present in subjects with chronic infection, independent of treatment and, in particular, STI. However, that the rapidly emerging single-point mutation M184V was detected in 14 of 25 subjects, compared with only 3 of 25 subjects harboring the slow-emerging L90M mutation ($P = .02$, Fisher's exact test), suggests that our results are causally linked to treatment and STI. The finding of a higher frequency of the M184V mutation, compared with the L90M mutation, is even more prominent if one considers the 20-times higher sensitivity of the L90M assay. Furthermore, both mutations have not been detected in plasma samples obtained from these subjects at time points before they received their first ART.

In conclusion, drug-resistant viruses were detected as minor populations of HIV-1 in the majority of subjects undergoing STI. It is not yet known whether a similar distribution occurs in subjects who stop successful therapy for longer periods without consistent reintroduction of HAART. However, our data suggest that such variants can emerge during periods of increased HIV-1 replication, when drug concentrations may be suboptimal. We observed tremendous variation in the appearance and disappearance, as well as in the timing of the emergence, of these mutations. On the basis of these observations, we suggest that STI should remain restricted to controlled clinical trials, to minimize the risk of the development of drug-resistant HIV-1 variants. The influence of minor populations of drug-resistant variants on long-term treatment is currently under investigation.

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