

Establishment of New Transmissible and Drug-Sensitive Human Immunodeficiency Virus Type 1 Wild Types due to Transmission of Nucleoside Analogue-Resistant Virus

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Sequence analysis of human immunodeficiency virus type 1 (HIV-1) from 74 persons with acute infections identified eight strains with mutations in the reverse transcriptase (RT) gene at positions 41, 67, 68, 70, 215, and 219 associated with resistance to the nucleoside analogue zidovudine (AZT). Follow-up of the fate of these resistant HIV-1 strains in four newly infected individuals revealed that they were readily replaced by sensitive strains. The RT of the resistant viruses changed at amino acid 215 from tyrosine (Y) to aspartic acid (D) or serine (S), with asparagine (N) as a transient intermediate, indicating the establishment of new wild types. When we introduced these mutations and the original threonine (T)-containing wild type into infectious molecular clones and assessed their competitive advantage *in vitro*, the order of fitness was in accord with the *in vivo* observations: 215Y < 215D = 215S = 215T. As detected by real-time nucleic acid sequence-based amplification with two molecular beacons, the addition of AZT or stavudine (d4T) to the viral cultures favored the 215Y mutant in a dose-dependent manner. Our results illustrate that infection with nucleoside analogue-resistant HIV leads in newly infected individuals to mutants that are sensitive to nucleoside analogues, but only a single mutation removed from drug-resistant HIV. Such mutants were shown to be transmissible, stable, and prone to rapid selection for resistance to AZT or d4T as soon as antiretroviral therapy was administered. Monitoring of patients for the presence of new HIV-1 wild types with D, S, or N residues at position 215 may be warranted in order to estimate the threat to long-term efficacy of regimens including nucleoside analogues.

Since 1987, when antiviral drug therapy of human immunodeficiency virus type 1 (HIV-1) infection started with the use of zidovudine (AZT), drug-resistant viral mutants have rapidly emerged (3, 12). Due to the error-prone reverse transcriptase (RT), viral mutants are generated during every replication cycle (16). The mutational pathway is in essence the result of a stochastic process in which a single mutation appears more frequently than double or multiple mutations (13). A newly formed viral mutant that has a higher replicative capacity than its parent will overgrow the parent. This process of selection results in the appearance of mutants with the highest fitness or replicative capacity in their environment (7). When an antiretroviral drug is present, mutants with the highest level of resistance to the drug will have an advantage. As soon as antiviral therapy is stopped, the original virus, which is usually well adapted to an environment without the drug, will win the competition with the resistant viruses (1). However, a different situation occurs when a drug-resistant virus is transmitted to a previously uninfected and untreated person. Separated from the original drug-sensitive virus and lacking this dominant competitor in the new drug-free environment, the drug-resistant virus will serve as a new starting point for the evolution of

HIV-1. The process does not necessarily lead back to a virus with a genotype identical to that of the original, drug-sensitive virus, but the phenotype (i.e., replicative capacity) will adapt to be optimally fit in the new drug-free environment (7).

Of 74 new HIV-1 infections that occurred in the period 1992 to 1999, 8 were caused by viruses with mutations at positions associated with AZT resistance. After their transmission to new hosts, those with the resistance-conferring T215Y mutation in the RT gene showed rapid evolution to better-replicating, more-fit viruses that acquired novel residues at amino acid 215 of RT. The novel evolutionary pathway led to a viral phenotype adapted to a drug-free environment and having a replicative capacity similar to that of the original wild type. There was evidence that the new viruses were transmissible and rapidly converted by a single mutation to a drug-resistant phenotype when suboptimal antiviral therapy was introduced.

MATERIALS AND METHODS

Study population. The Amsterdam cohort studies (ACS) have monitored a cohort of homosexual men and a cohort of intravenous drug users, with quarterly screening of participants for the presence of the HIV-1 antigen and antibodies. New HIV-1 infections are identified by a seroconversion to the HIV-1 antigen and/or antibody with confirmation by Western blotting and HIV-1 viral load assay. In addition, patients admitted to the clinic of the Academic Medical Center (AMC) in Amsterdam with symptoms of suspected acute HIV-1 infection have been examined. We studied a total of 74 new infections identified by ACS and AMC between 1992 and 1999. Of these 74 new HIV-1 infections, 8 had RT mutations that are associated with AZT-resistance (see below for the sequencing method). Mutations specifically associated with RT inhibitors other than AZT (zalcitabine [ddC], didanosine, stavudine [d4T], lamivudine (3TC), nevirapine

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and other nonnucleoside RT inhibitors) were not found. Of the 28 new HIV-1 infections identified during or after 1996, the year of the introduction of protease inhibitors, none involved viruses that had mutations that confer resistance to protease inhibitors. Sequences of the 5' end of the RT gene with mutations associated with AZT resistance were deposited in GenBank (see below for sequence numbers).

Viral load and CD4 counts. Viral load was determined by using the NucliSens assay of Organon Teknika BV (Boxtel, The Netherlands). CD4⁺ cells in blood were counted by standard flow cytometry using a FACScan flow cytometer (Becton Dickinson, San Jose, Calif.) and commercially available monoclonal antibodies (Becton Dickinson).

Screening for resistance-conferring mutations. Viral RNA was isolated from 200 µl of serum drawn from each of the 74 subjects at the first point of seroconversion to the HIV-1 antigen or antibody by using the method described by Boom et al. (2). Part of the HIV-1 *pol* region including the part of the RT gene encoding the amino terminus was amplified by RT-PCR, essentially according to the protocol described by Nijhuis et al. (18). To facilitate accurate two-stranded sequence analysis of the amino terminus-encoding part of the RT gene, we adapted the nested PCR in the Nijhuis protocol by amplifying two overlapping fragments instead of one. The 5' fragment was amplified using primer SP6-RT19new, *ATTAGGTGACACTATAGCACCTGTCAACATAATTGGAAG* (SP6 sequence is in italics; HXB-2 nucleotides [nt] 2491 to 2512), and T7-B-SEQ, *TAATACGACTCACTATAGGGAATATTGCTGGTGATCCCTTTCCA* (T7 sequence is in italics; HXB-2 nt 3030 to 2006). The 3' fragment was amplified with primers SP6-C-SEQ, *ATTAGGTGACACTATAGGTATACTGCATTTACCA TACC* (SP6 sequence is in italics; HXB-2 nt 2927 to 2947), and T7-ET10, *TAA TACGACTCACTATAGGGCTGCCAGTTCTAGCTCTGCTTC* (T7 sequence is in italics; HXB-2 nt 3462 to 3441). Both strands of the nested PCR fragments were directly sequenced using the SP6 and T7 primer sequences. Sequencing was performed with *Taq* dye primers (Applied Biosystems, Foster City, Calif.) and the ThermoSequenase fluorescence-labeled primer cycle sequencing kit (Amersham International, Little Chalfont, England). The sequence products were analyzed on an automatic sequencer (Applied Biosystems DNA sequencer model 373A stretch or 377). The sequence of the RT gene was screened at positions associated with drug resistance as described by Hirsch et al. (10). For new infections caused by viruses with mutations at positions associated with AZT resistance, serum samples drawn after the seroconversion point were analyzed for changes compared to the seroconversion sample. Mixtures of different viral RT sequences were quantified by assessing the ratio between the relevant peak areas of the signals of the corresponding nucleotides on the electropherogram using the sequences of both strands. In cases identified during or after 1996, the year of introduction of protease inhibitors in The Netherlands, we sequenced the protease gene as well as the RT gene. The protease gene was amplified according to the procedure described by de Jong et al. (5). Nested PCR was performed by using primers SP6-A-SEQ, *ATTAGGTGACACTATAGGAGCCAACAGCCC CACCAG* (SP6 sequence is in italics; HXB-2 nt 2149 to 2167) and T7-A-SEQ, *TAATACGACTCACTATAGGGTAAAGAAGACAGTTACCG* (T7 sequence is in italics; HXB-2 nt 2639 to 2621). Sequence analysis was performed as above for the RT gene.

Infectious molecular clones. Biological clones of the virus of patient 4 were prepared by a limiting-dilution series of human peripheral blood mononuclear cells (PBMC) derived from a sample taken 1 month after seroconversion (14). Viral RNA was isolated from the culture supernatants of individual biological clones. The complete RT gene of the virus was reverse transcribed and amplified (5) but by using primers ET44 (*GACATTTATCACAGCTGGCTAC*; antisense; nt 4338 to 4359 of HIVHXB2CG; GenBank accession no. K03455) and 5'-PROT-OUT (*GAGCCAACAGCCCCACCAG*; nt 2149 to 2167; sense) in the RT reaction and first PCR and by using primers ET42 (*CTGTTGACTCAGAT TGGTTGCACTTTAAATTTTCC*; nt 2517 to 2551; sense) and ET17 (*AGGT GGCAGGTTAAAATCAACTAGCCATGCCATTGCTCTCC*; nt 4319 to 4285; antisense) in the nested PCR. The PCR product was cloned in plasmid pCR-2.1 using a TA vector system (InVitrogen BV, Groningen, The Netherlands). Sequence analysis of the 5' end of the RT gene was performed, and a clone with the T215Y mutation (AZT resistant) was chosen. The insert was subcloned in the *EcoRI* site of vector pUC19 to remove the flanking *BstXI* sites, creating p4-Y. PCR-mediated mutagenesis of amino acid 215 of RT was performed essentially as described by de Jong et al. (5) except that primer ET10 (*CTGCGAGTTCT AGCTCTGCTTC*; nt 3462 to 3441) and the sense mutagenesis primer for the first PCR were used, whereas primer ET07 (*GGAAAGTTCAATTAGGAATA CC*; nt 2812 to 2832) and the antisense mutagenesis primer were used for the second PCR. The two PCR products were combined, and a third PCR was performed using primers ET10 and ET07. The resulting PCR product was digested with *BstXI* and cloned into a *BstXI*-digested p4-Y. Mutagenesis primers

ET47 (*GAAGTGGGGATTTCGACACACCAGACAAAAAAC*; nt 3170 to 3209; sense; codon 215 is underlined) and ET48 (*GTTTTTTGTCTGGTGTGT CCGAATCCCCACTTC*; nt 3209 to 3170; complementary) were used to introduce the 215 aspartic acid (GAC codon) mutation; ET49 (*GAAGTGGGGAT TCCTCCACACCAGACAAAAAAC*; sense) and ET50 (*GTTTTTTGTCTGGT GTGGAGAATCCCCACTTC*; complementary) were used to introduce the 215 serine (TCC codon) mutation; ET51 (*GAAGTGGGGATTCCACACACAG AAAAAAAC*; sense) and ET52 (*GTTTTTTGTCTGGTGTGGTGAATCCCC ACTTC*; complementary) were used to introduce the 215 threonine (ACC codon) wild-type mutation. Sequencing was performed to verify that the exchanged *BstXI* fragment contained the introduced mutations. By this procedure, we created plasmids p4-D, p4-S, and p4-T encoding the indicated (one-letter code) amino acids at the 215 position.

At week 154, viral RNA was isolated from a sample drawn from patient 4, and RT-PCR was performed as described above using primers ET42 and ET10; the product was cloned in the TA cloning vector pCRII (InVitrogen BV). Sequence analysis showed that individual clones were representative of the quasispecies that contained an S68G change relative to the p4-Y clone. In addition, these clones had naturally occurring variations at amino acids 39 and 135 (T39A, I135T). The 3' end of one of these clones (p4-68G) starting from the *SspI* site (HXB-2 nt 3025) was replaced by the 3' end of p4-Y or p4-D creating p4-Y-68G and p4-D-68G, respectively. The infectious molecular clone in which RT genes derived from patient 4 were recombined was pHIV-Lai, lacking its RT gene (pLAI-ART). The RT gene deletion was obtained by PCR-mediated mutagenesis. Briefly, the deletion was constructed in a subclone of HIV-Lai containing the *ApaI*-*NcoI* fragment by using primers ET40 (*TGACCGCGGAAAATTTA AAGTGCAAC*; nt 2550 to 2532; antisense; *SacII* site underlined) and ET23 (*AGTCCCGGAGAGCAATGGCTAGT*; nt 4286 to 4300; sense; *SacII* site underlined) and primers 5' and 3' of *ApaI* and *NcoI* sites, respectively. This created molecular clone pLAI-ART with a *SacII* site replacing the RT gene.

Recombinant viruses with a recipient-derived RT gene. To obtain recombinant viruses, C33A cells (human cervix carcinoma cell line) were seeded in 24-well plates and grown to 80% confluence in 1 ml of RPMI 1640 medium (Life Technologies, Breda, The Netherlands) supplemented with 10% fetal calf serum and antibiotics. Cotransfection of 300 ng of pLAI-ART and 200 ng of the purified RT gene-containing *EcoRI* fragment of p4-Y, -D, -S, -T, -Y-68G, or -D-68G was performed using the TFX-50 reagent protocol (Promega Benelux BV, Leiden, The Netherlands). One day after transfection, 200,000 MT2 cells per well were added, and C33A and MT2 cells were cocultured. After 2 days, the MT2 cells including medium were transferred to a 25-cm² flask, with the addition of fresh medium and MT2 cells. Cells were monitored for the formation of syncytia, and the supernatant containing the recombinant virus was harvested after the spread of syncytia throughout the culture. The virus was frozen into aliquots at -70°C. The virus titer (50% tissue culture infective dose [TCID₅₀]) was determined by limiting-dilution titration on MT2 cells. Resistance to antiviral drugs was assayed by VIRCO (Edegem, Belgium) (9).

Competition experiments were performed by mixing a total amount of 1,000 TCID₅₀ of the recombinant viruses in different ratios. These virus mixtures were used to infect 2 ml of phytohemagglutinin-stimulated PBMC (2 × 10⁶ cells/ml), which were maintained in a six-well plate in RPMI 1640 medium supplemented with 10% fetal calf serum and interleukin-2 (6). Twice a week, cells were passaged, and 10 µl of the culture was transferred to 2 ml of a new culture of 2 × 10⁶ PBMC/ml. Similarly, 500,000 MT2 cells were infected with 1,000 TCID₅₀ and maintained in 1 ml of RPMI medium supplemented with 10% fetal calf serum in a 24-well plate. When syncytia in the MT2 cells had formed, 5 µl of the culture was transferred to 1 ml of a new culture composed of 1 ml of medium containing 500,000 MT2 cells. Competition experiments were performed in the absence or presence of antiviral drugs (i.e., AZT, d4T, or ddC). At each passage, samples from the supernatant from which to isolate viral RNA for sequence analysis were drawn. Viral RNA was isolated according to the method of Boom et al. (2). The RNA was directly sequenced as described above. The ratios between the different recombinant viruses were determined from the direct sequence by assessing the ratios between the relevant peak areas of the signals on the electropherogram corresponding to the nucleotides encoding amino acid 215 of RT. Alternatively, the ratios between the different viruses were determined by using real-time nucleic acid sequence-based amplification (NASBA) with molecular beacons, as described in the next section.

Quantification of mutant mixtures using real-time NASBA and molecular beacons. RNA was isolated from 100 µl of culture supernatant according to the method of Boom et al. (2). The nucleic acids were eluted in 50 µl of water, and 5 µl of the eluate was used as input for amplification by NASBA. NASBA was performed by using the basic kit of Organon Teknika supplemented with primers 215-P2 *GACTTAGAAATAGGGCAGCA* (HXB-2 nt 2705 to 2724) and 215-P1

TABLE 1. Example of the fitness calculation with data from patient 1

Sample	Days ^a	Virus load ^b (copies/ml)	% of virus:	
			215Y	215N
1	47	400	100	0
2	299	400	80	20
3	392	230	33	67
4	517	620	9	91
5	614	7,200	7 ^c	80 ^c
6	694	1,400	0	100

^a Days after seroconversion.

^b RNA load in serum.

^c The mixture contained 13% 215D virus.

AATTCTAATACGACTCACTATAGGGGTTTCATAACCCATCCAAAGGAAT GGA (T7 sequence in italics; HXB-2 nt 2831 to 2806) and the molecular beacons (20) RT 215GAC, cegactcTCGACACACCAGACAAAAACgagtcgg (FAM, dabcyI), and RT 215TAC, ccgactcTCTACACACCAGACAAAAACgagtcgg (6-ROX, dabcyI) (stem sequence is in lowercase; HXB-2 nt 2772 to 2792; position 215 [underlined, boldface] is either a GAC [D] or TAC [Y] codon). The two primers and two beacons were each added to a final concentration of 200 nM. Fluorescence was measured in real time on a Fluoroscanner Ascent (Labsystems Oy, Helsinki, Finland). The assays were calibrated by mixing different quantities of in vitro-synthesized RNA containing the GAC or TAC position 215 codon. To synthesize the RNA, PCR fragments were generated from p4-D-68G and p4-Y-68G by using primers AATTCTAATACGACTCACTATAGGG (T7 site located in the plasmid) and ET10 (HXB-2 nt 3462 to 3441). These PCR fragments were used to produce RNA containing the GAC or TAC position 215 codon by using T7 RNA polymerase (Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands) in an in vitro transcription reaction. Under our reaction conditions with 10⁵ molecules of in vitro-synthesized RNA per reaction, the beacon specific for the GAC codon did not react with the RNA containing the TAC codon and vice versa. In vitro-synthesized RNA containing the GAC and TAC codons was mixed at different percentages (0, 2, 4, 10, 33, 50, 67, 90, 96, 98, and 100%). Repeated (*n* = 5) experiments showed that quantification of the mixtures using real-time NASBA could be achieved until 4% of either the TAC or GAC variant (see Fig. 4A and B). Below 4% of the variant, the variant could be observed but not quantified. Quantification of mixtures by real-time NASBA and direct sequencing were compared for the competitions in the presence of AZT (see Fig. 4C). The methods gave comparable percentages (±10%) of a variant mixture, provided that direct sequencing was performed using the ET dye primer on the ABI automatic sequencers and not the big-dye techniques on the ABI machines. However, when a variant was present in small amounts (less than 10%), quantification by direct sequencing was no longer reliable due to background signals. Real-time NASBA was broader in dynamic range; it could reliably quantify a variant at a level as low as 4% and could detect a variant at a level as low as 1%.

Fitness calculation. Selection coefficients were calculated by a novel method involving the estimation of the total viral replication during the observation period (16a). The method can be used both for steady-state viral populations, to which most previously used methods apply (8), and for decreasing or expanding viral populations, such as those observed, for example, in cell cultures. Briefly, the model is derived as follows. Because mutations in the RT are expected to mainly influence the replication rate of a mutant virus and to hardly influence its half-life, we write $dW/dM = rW - \delta W$ and $dM/dt = r(1 + s)M - \delta M$ for the dynamics of wild-type virus *W* and mutant virus *M*. Here *r* is the (wild-type) replication rate (which may change over time [*t*]), 1/δ is the generation time, and *s* is the classical coefficient of selection. Because this model combines the dynamics of productively infected cells and free virions, the generation time should be about 2 days (11). The same model has been used before for estimating *s* from in vivo data with approximately steady-state viral loads (8). One conventionally defines the frequency of the mutant genotype by the equation $P = M/(W + M)$, and hence $1 - p$ is the frequency of the wild-type virus. If *r* is a constant, one writes the solutions $W(t) = W(0)e^{(r - \delta)t}$ and $M(t) = M(0)e^{(r(1 + s) - \delta)t}$ so that *s* is computed from the logarithms of the genotype ratios ($H = M/W = p/[1 - p]$) at time 0 and time *t*, i.e., $s = \ln[H(t)/H(0)]/rt$.

Thus, for estimating the relative fitness one generally needs to know the ratio of the genotype frequencies and the total replication, *rt*. *r* may vary over time, however. This requires the integration of the replication rate over the experiment. Fortunately, *s* can still be estimated by the simple formula (16a) $s = \ln$

$[H(t)/H(0)]/\{\ln[W(t)/W(0)] + \delta t\}$, which is available at <http://www.binf.bio.uu.nl/~rdb/fitness.html>. If the total viral loads and the percentages of wild-type virus at times 0 and *t* are known, the website equation can be used to compute the change in the wild-type virus load, $W(t)/W(0)$, and the change in the ratio of the genotype frequencies, $H(t)/H(0)$, to give *s*. *H* is undefined when the percentage of mutant virus is estimated as 0 or 100%. Whenever possible, we therefore ignored such data points. However, in a few instances (patients 2 and 4) no alternative data points were available. In such instances, we took 1 or 99% for the genotype frequency, which was a conceivable assumption (see Table 1 for the example of patient 1). For the fitness calculations for the viral culture we took the cumulative p24 value as the viral load, i.e., a p24 value of 1,000 pg/ml after a 1:100 dilution of a viral culture was 100,000 pg/ml, allowing us to keep a dilution factor of 1 in the formula on the website.

We calculated *s* over the interval between samples 1 and 6 (Table 1) (using 99 and 1% for the percentages of virus with Y at position 215 [215Y virus] in samples 1 and 6, respectively); the fitness difference between 215Y and 215N was 2.8%. Corresponding fitness differences were 2.3, 3.3, 2.6, 2.5, and 3.0% for intervals between samples 2 and 5, 2 and 4, 2 and 3, 3 and 4 (all of which used detected percentages of 215Y virus), and 2 and 6 (which used 1% 215Y virus for sample 6), respectively. The one-sample *t* test of the SSPS, version 8.0, statistical package was used to calculate the mean and the 95% confidence interval. We concluded that 215N had a relative fitness of 103% (95% confidence interval: 102 to 104%), with 215Y fitness = 100%.

We calculated *s* over the interval between samples 1 and 4 for the virus culture of the competition between 215Y and 215T viruses (Table 2); the fitness difference between 215Y and 215T was 5.1%. Corresponding fitness differences were 5.4, 6.0, 4.7, and 4.9% for intervals between samples 2 and 4, 3 and 4, 1 and 3, and 2 and 3, respectively. We concluded that 215T had a relative fitness of 105% (95% confidence interval: 104 to 106%), with 215Y fitness = 100%.

Nucleotide sequence accession numbers. Accession numbers for viral RT gene sequences obtained in this study were as follows: patient 1 (H0671), AF265569; patient 2 (I6056), AF265572; patient 3 (H0095), AF265570; patient 4 (I7052), AF265571; patient 5 (H0137), AF265568; patient 6 (I3234), AF265573; patient 7 (M12690), AF265574; patient 8 (2202575), AF265567.

RESULTS

In vivo evolution of viruses with a mutated RT gene in the absence of an antiviral drug. Among 74 new HIV-1 infections occurring between 1992 and 1999, 8 were found to be due to viruses that have mutations in the RT gene that are associated with AZT resistance. At seroconversion, the corresponding mutations in RT were M41L and T215Y (patients 1, 2, and 4); T215Y (patient 3); D67N, K70R, and T215F (patient 5); D67N, K70R, and K219Q (patient 6); M41L and the T215D mutation, which involved an unusual amino acid not seen associated with AZT resistance (patient 7); and K70R (patient 8). The K70R mutation in the virus infecting patient 8 could be the result of a natural polymorphism, although in the absence of AZT the K70R variant rarely becomes the dominant variant in the viral quasispecies (17). The eight infections were monitored until antiviral therapy (Fig. 1). Infections 1 to 5, which all were found at seroconversion to have an AZT resistance-conferring mutation at amino acid 215 of RT (either 215Y or

TABLE 2. Example of the fitness calculation with the virus culture of the competition between 215Y and 215T viruses

Sample	Days ^a	Virus load ^b (ng/ml)	% of virus:	
			215Y	215T
1	3	69	76	24
2	10	4.0 × 10 ⁵	65	35
3	24	1.1 × 10 ¹¹	41	59
4	35	4.5 × 10 ¹⁴	23	77

^a Days after infection.

^b Cumulative p24 in culture medium.

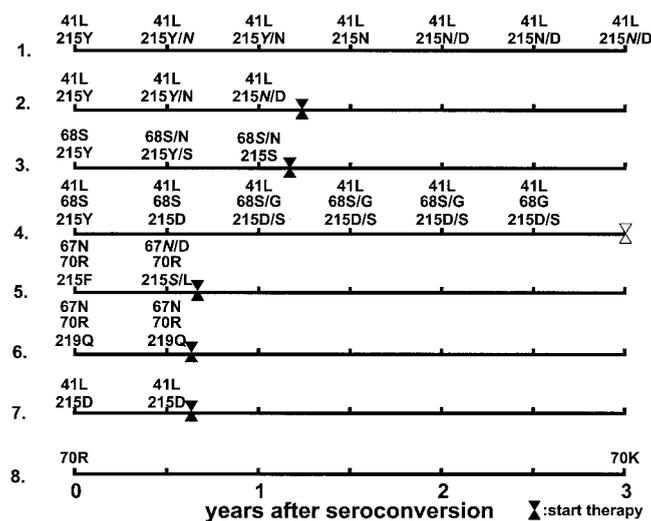


FIG. 1. In vivo evolution of transmitted viruses with drug resistance-conferring mutations in the absence of antiretroviral therapy. Indicated are the positions and amino acids (minor species in italics) in which the viruses differ from the wild type at positions known to be involved in drug resistance (10). Our subjects were monitored until antiviral therapy.

215F) showed evolution in their RT genes. Since infections 1 to 4 could be monitored for at least 1 year before antiviral therapy was given, the evolution of the RT gene, and in particular the evolution at amino acid 215 of RT, was analyzed in these cases (Fig. 2A, 1 to 4).

In patient 1 at seroconversion, the RT of the infecting viruses had the M41L and T215Y mutations conferring AZT resistance. While the codon at position 41 of the RT gene did not change, amino acid codon 215 changed from TAC, encoding tyrosine, to AAC, encoding asparagine. The 215N virus had completely replaced 215Y within 100 weeks (2 years) after seroconversion. Over the next 50 weeks, the AAC codon at position 215 was gradually replaced by a GAC codon encoding aspartic acid, which remained the dominant species thereafter.

In patient 2 at seroconversion, the M41L and T215Y mutations were likewise present and the mutants evolved as in patient 1, but on a far smaller time scale. During the 36 weeks that patient 2 was monitored 215Y evolved to a mixture of 215N and 215D, in which 215D eventually became the dominant species, a process that took 150 weeks in patient 1.

In patient 3, RT had only the T215Y mutation at seroconversion, with codon 215 evolving from TAC (215Y) to AGC, encoding serine (215S), although at week 11 GAC (215D) was transiently observed. Unlike the other mutations we studied, which involve a one-nucleotide change within the codon 215, the change from TAC to AGC involved a two-nucleotide change, and no obvious intermediate was detected. However, in this infection, the 215 AAC codon may have been transiently formed as an intermediate to the 215 AGC codon encoding serine. A notable mutation was observed at codon 68 of the RT gene, which changed from wild-type AGC (serine) to a mixture of AGA and AAC (asparagine).

In patient 4, RT had the M41L and T215Y mutations at seroconversion. At week 3 after seroconversion, the 215 TAC (Y) codon was already being replaced by the 215 GAC (D)

codon, with full replacement observed at week 20. At week 37, we detected a mixture of the 215 GAC codon and the 215 TCC codon (S), in which the relative abundances of the two fluctuated until week 150, when the last sample was drawn before therapy began. The changes to an aspartic acid and to a serine at position 215 of RT required only a single mutation, from the TAC codon to GAC and TCC codons, respectively. Analogous to what was observed in patient 3, the RT gene of patient 4 showed a change at position 68. In this case, however, the wild-type 68 AGC codon (serine) changed to a GGC codon encoding glycine (68G). At week 120, wild-type 68S had been completely replaced by 68G, which persisted thereafter. An overview of the mutational pathways in the different patients is shown in Fig. 2B.

The rate of RT evolution in these cases varied considerably. In patient 1, replacement of 215Y by 215N and subsequently by 215D took place over a time period of more than 150 weeks. Concurrent with the replacement of 215Y by 215N, the viral load increased from below 1,000 copies/ml to 10,000 copies/ml, and it increased further to 30,000 copies/ml when 215D became the dominant species. During this period, the CD4 T-cell counts remained stable and high at approximately 1,000/ μ l. In patient 2, replacement of 215Y by 215D occurred within less than 40 weeks, with a viral load between 30,000 and 100,000 copies/ml and CD4 T-cell counts hovering between 400 and 600/ μ l. In patient 3, replacement of 215Y by 215S occurred after 40 weeks; the viral load declined from 300,000 copies/ml at seroconversion to 10,000 to 30,000 copies/ml from week 30 to 55 and CD4 T cells remained at 200 to 400/ μ l. In patient 4, replacement of 215Y by 215D occurred within 25 weeks, with a viral load that declined from above 100,000 copies/ml at seroconversion to about 25,000 copies/ml at week 100 and with CD4 T-cell counts varying from approximately 300 to 600/ μ l.

In all three patients who had been infected with a virus in which the RT gene contained both the 41L and 215Y mutations, the dominant viral species that emerged contained an RT with the 41L and 215D mutations. Patient 7 was infected with a virus containing an RT with these 41L and 215D mutations, indicating that a virus with these mutations is transmissible.

In vivo fitnesses of the newly emerged viruses with mutations in the RT gene. The increased replicative capacity, or fitness, of the newly emerged viruses with changes in their RT genes was expressed relative to the replicative capacity of the 215Y variant at seroconversion, which was arbitrarily set at 100%. The fitnesses of the variants newly formed in an environment without an antiviral drug were calculated. In patient 1 the available serum samples allowed the calculation of the fitness of the 215N variant, which was 103% (95% confidence interval: 102 to 104%) of that of 215Y in an environment without an antiviral drug. By the same method, the fitness of the 215S variant (patient 3) was calculated to be 106% (103 to 109%) of that of 215Y and the fitness of the 215D variant was calculated to be 107 (patient 2) or 110% (patient 4) (96 to 124%) of that of the 215Y variant. By way of comparison, the S68G change observed in the RT of patient 4 resulted in a relative fitness of the 68G viruses of 101% compared to that of the 68S virus, which was set at 100%. To give an impression of the competition between the new variants and the AZT-resistant 215Y, we plotted the fractions (relative to 215Y) of the

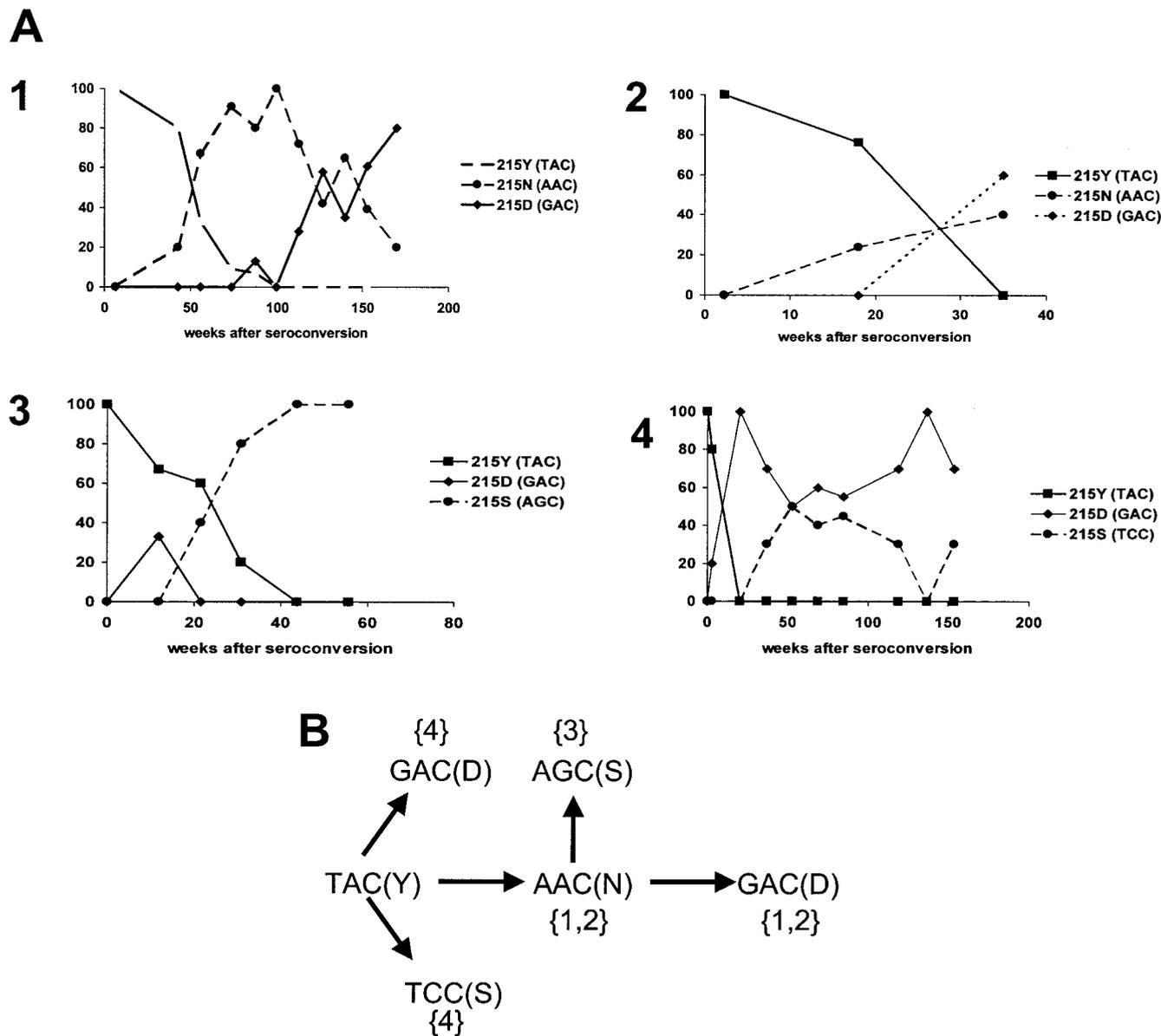


FIG. 2. In vivo evolution at amino acid 215 of RT. (A) Relative abundances of viruses with the indicated amino acids at position 215 of RT after transmission of AZT-resistant viruses. The evolution of transmitted viruses is shown in patients 1 to 4. All start with 215Y and evolve to 215D (patients 1, 2, and 4) and/or 215S (patients 3, and 4), with 215N observed as an intermediate (patients 1 and 2). (B) In vivo mutational pathways as observed (patients 1, 2, and 4) or hypothesized (patient 3) at amino acid 215 of RT after transmission of AZT-resistant viruses.

different variants in the viral population (on a log scale) against the elapsed time after seroconversion (Fig. 3A).

In vitro fitnesses of viruses differing at amino acid 215 of RT. In vivo, if HIV-1 isolates found in two different samples drawn from a patient show genetic differences, they differ usually at multiple positions, of which only a few may significantly contribute to a change in replicative capacity. To examine the significance of RT amino acid 215 for replicative capacity, viruses that differed only at this amino acid were constructed. In the RT isolated from patient 4, containing the M41L and T215Y mutations, only the 215 position was changed by in vitro mutagenesis to either 215D, 215S, or to 215T, the wild-type amino acid present before the introduction of AZT. The wild-

type 215T was found also in the viruses of the donor of patient 4 before he received AZT and resistant viruses developed. The various RT genes were recombined into an HIV-1 Lai background, creating viruses that differed only at amino acid 215 of RT. Competition between the resulting viruses was observed in PBMC in the absence of an antiviral drug. In that environment, the 215Y virus was the least fit virus. The relative fitnesses of all the viruses were calculated. The in vitro relative fitnesses of 215D, 215S, and 215T compared to that of 215Y (100%) were 104 (95% confidence interval: 102 to 106%), 107 (104 to 109%), and 105% (104 to 106%), respectively (Fig. 3B). Competition among the 215D and the 215S or 215T viruses showed them to be equally fit, i.e., having a fitness difference of less

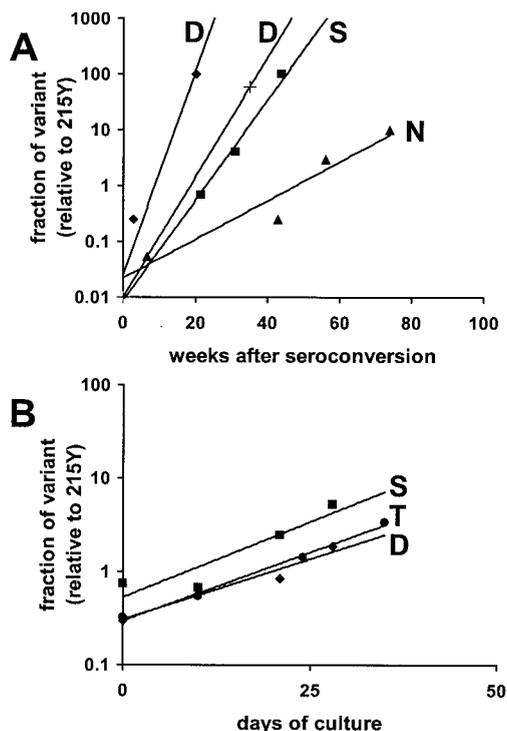


FIG. 3. In vivo and in vitro relative fitnesses of viruses marked by their amino acids at position 215 of RT. (A) Fitness plot of viruses differing at amino acid 215 (N, S, and D), which was derived from the observed replacement of 215Y by viral variant 215N, 215S, or 215D in the four patients (two of them had a 215D). The data points correspond to the observed viral mixtures in the patients (Fig. 2A). Shown are the rates (weeks after seroconversion) at which and to what extent (on a log scale) 215Y virus is replaced by a different variant. (B) Fitness plot of viruses differing only at amino acid 215 of RT, which competed in PBMC in the absence of an antiretroviral drug. Shown are the rates (number of days of culture) at which and to what extent (on a log scale) variants 215D (◆), 215S (■), and 215T (●) replaced 215Y. When 215D was competed with 215T or 215S, 215D was found to be as fit as 215T or 215S.

than 1%. The order of in vitro fitness of the viruses differing only at amino acid 215 was 215Y < 215D = 215S = 215T, corresponding to the in vivo-observed order.

Characterization of the viruses after antiviral therapy by using real-time NASBA. Patient 1 was not treated because his CD4 counts remained stable and high at approximately 1,000 CD4 cells/ μ l. Patients 2 and 3 were treated with a combination of protease and RT inhibitors (indinavir plus ritonavir plus d4T plus 3TC and indinavir plus d4T plus 3TC), and their viral loads declined to below detection level (<400 copies/ml). Patient 4 was treated at 160 weeks after seroconversion with RT inhibitors d4T and ddC. No change in the viral load was observed between week 154 (pretherapy) and week 170 (after therapy), indicative of the development of resistance to the antiviral drug. In this case, the only observed mutation in RT was at amino acid 215, which was changed from a mixture of 215D and 215S to 215Y by a single mutation (GAC and/or TCC to TAC). Phylogenetic analysis of the RT genes of the viruses found before and after therapy showed that the post-therapy 215Y virus was derived from viruses present before therapy but not present at transmission. The newer viruses shared 215Y with the transmission virus but had, in addition,

mutations at positions 68 (S68G), 39 (T39A), and 135 (I135T). To examine whether the change of environment caused by the therapy with d4T and ddC was involved in the selection of a viral population with the 215D- or 215S-to-215Y mutation, viruses that differed only at amino acid 215 of RT were studied. The viruses tested had either 215D or 215Y with 68G (present directly before and after therapy) or 68S (present directly after transmission). By using competition experiments with MT2 cells, the replicative capacities of the viruses were compared at increasing concentrations of AZT, d4T, or ddC. To facilitate the analysis of the competition experiments, we developed a real-time NASBA with two molecular beacons differing at one nucleotide. The dynamic range of quantification and the throughput of the real time NASBA are higher than those in sequence analysis, which generally cannot quantify mixtures with less than 10% of a variant. The molecular beacons, among which the GAC beacon contained a FAM label and the TAC beacon contained a ROX label, were able to discriminate viruses differing at the 215 codon of the RT gene (Fig. 4A). The real-time NASBA was able to quantify mixtures containing as little as 4% of either the GAC- or TAC-containing virus (Fig. 4B) and to detect the GAC- or TAC-containing virus in a mixture in which they composed no more than 1%. Using the real-time NASBA, the analysis of the competition experiments showed that the background of the viruses with the 68S or 68G mutation (either those directly after transmission or those present at therapy) did not influence the phenotype. However, the 215D-to-215Y change was crucial to obtain a replicative advantage in the environment with both AZT and d4T (Fig. 4C). The replicative advantage of a virus with a 215Y codon in the presence of AZT or d4T varied in a dose-dependent manner. The replicative advantage of 215Y virus could be expressed as an average fitness difference of 5 and 11% in the presence of 45 and 170 nM AZT, respectively (0.5 and 2 times, respectively, the 50% inhibitory concentration [IC_{50}] of a sensitive virus), and 2 and 28% in the presence of 3 and 12 μ M d4T, respectively (0.5 and 2 times, respectively, the IC_{50} of a sensitive virus), indicating that the replicative advantage of 215Y increased with higher drug concentrations. At 1.5 and 4.5 μ M (0.5 and 1.5 times, respectively, the IC_{50} of a sensitive virus), ddC did not appear to influence the relative replicative capacities of the viruses. In classical phenotypic resistance assays, the 215Y virus is >100-fold more resistant to AZT and 2-fold more resistant to d4T than the wild type and sensitive to ddC, whereas the 215D and 215S viruses are sensitive to AZT, d4T, and ddC.

DISCUSSION

Our study indicates that between 1992 and 1999, 10 to 15% of new HIV-1 infections in Amsterdam, The Netherlands, were caused by HIV-1 strains with mutations in the RT gene at positions that are associated with AZT resistance. We found no mutations associated with resistance to other antiretroviral drugs, including the protease inhibitors introduced in 1996. When the HIV-1 strains with drug resistance-conferring mutations are transmitted, a new environment without antiviral drugs in a new host is encountered. After transmission of usually a small amount of virus, the wild-type strains from which the drug-resistant strains were derived are lost from the

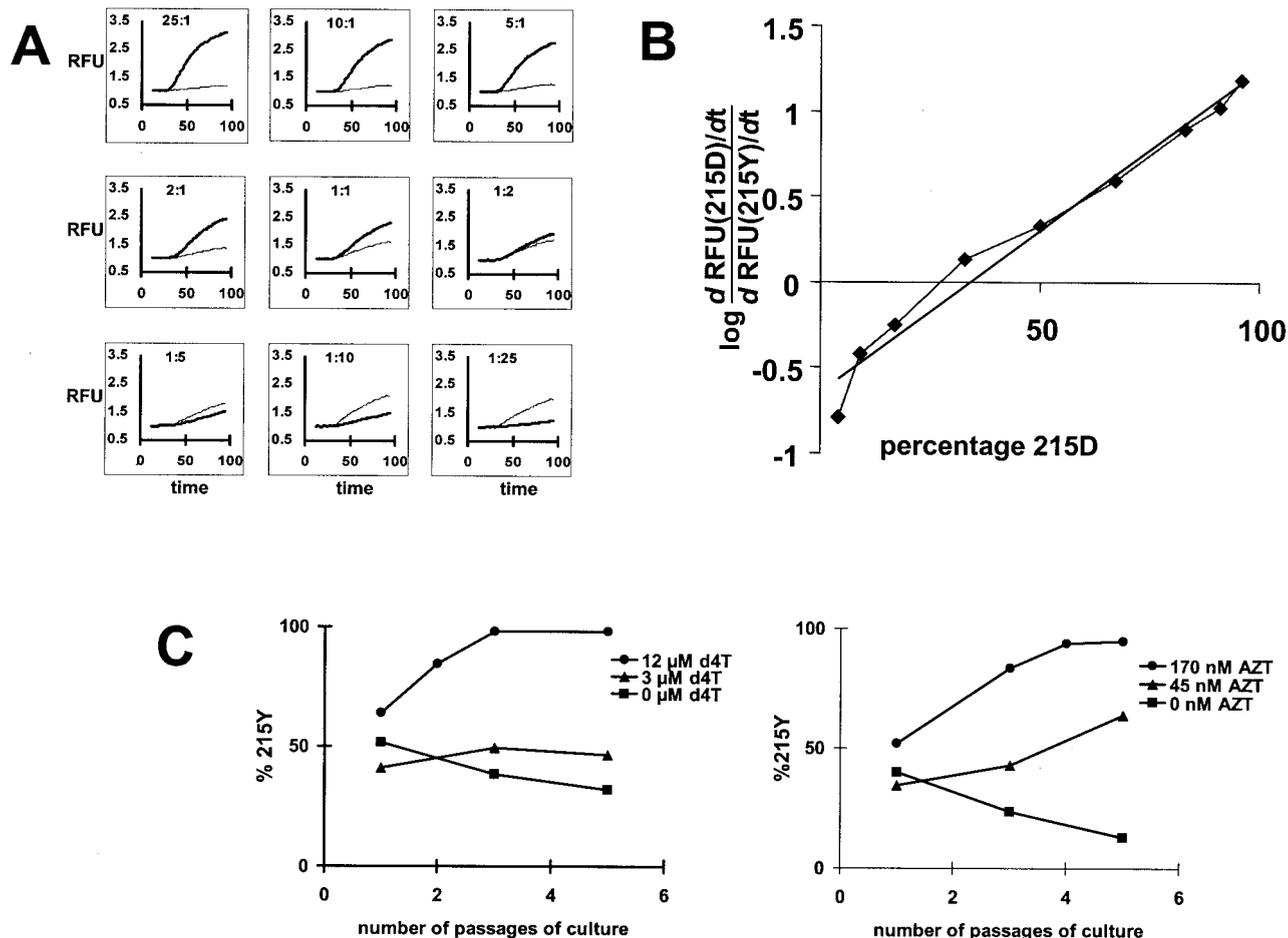


FIG. 4. In vitro competitions of 215Y and 215D viruses in the absence and presence of antiviral drugs as analyzed by using real-time NASBA. (A) Representative experiment in which relative fluorescence units (RFU) are shown as a function of time when RNA mixtures (total of 10^5 molecules) of 215D and 215Y (ranging from a 25:1 to a 1:25 ratio) were assayed by real-time NASBA using GAC (FAM-labeled) and TAC (ROX-labeled) molecular beacons indicative of 215D and 215Y, respectively. (B) Calibration curve derived from panel A. The log function of the slope of the increase of fluorescence in time is proportional to the relative abundances of 215D and 215Y in a sample, which can be inferred from the known mixtures. (C) Competition between 215Y and 215D viruses in the presence of increasing concentrations of d4T (left) or AZT (right). Viruses containing RT with 215Y and 215D competed in MT2 cells in the presence of 0, 3, or 12 μ M d4T or 0, 45, or 170 nM AZT. The ratio of 215Y/215D virus in the supernatant at passage was determined by using real-time NASBA.

(memory of the) quasispecies and thereby are excluded from competition in the new environment (19). The separation of individual viruses from the remainder of competing viruses by bottleneck transmission is reminiscent of the founding of an island population and may lead to new directions in evolution (15). In the new, drug-free host, viruses that have an increased replicative capacity or fitness compared to that of their drug-resistant parent will be selected. Although the replicative properties of the viruses we studied will most likely be determined by more than one gene, we focused on the resistance-conferring RT gene. We found that the drug-resistance-conferring RT amino acid 215Y in viruses changed after transmission, but not to the wild-type threonine. Instead, novel amino acids at position 215 were observed (see also reference 21). Single mutations in the TAC codon (215Y) gave rise to AAC (asparagine), GAC (aspartic acid), or TCC (serine). In addition, in one of the new infections an AGC codon (serine, 215S) was formed by a two-step mutation. Selection occurred for the fittest variant, the 215N virus being less fit than the 215D or

215S virus. Compared to the transmitted AZT-resistant 215Y virus the 215N viruses were 3% more fit, the 215S viruses were 6% more fit, and the 215D viruses were 7 to 10% more fit. Our calculations assumed a constant viral generation time; however, variation among the respective infections could affect the relative fitness values. In this respect, patient 1, with an initially low viral load and slow replacement of the different variants, is illustrative. Nevertheless, the order of viral fitness is 215-tyrosine < 215-asparagine < 215-aspartic acid = 215-serine. In vitro experiments in a drug-free environment showed that wild-type viruses with 215-threonine were as fit as the 215D or 215S viruses and fitter than the 215Y viruses. Threonine (ACC codon; 215T) can be formed by a two-step mutational process from the TAC codon (215Y). Such a two-step mutant, generated by stochastic mutational processes, will usually be formed later than one-step mutants such as 215D (GAC) and 215S (TCC). The principle of competitive exclusion could explain why we observed no 215T viruses in the new infections started with AZT-resistant viruses, since an equally fit variant will not

replace an existing virus that already dominates the population (4). In this group, the phenotype of a 215D or 215S virus will to a large extent be the new wild type. This is most clearly shown in the virus of patient 7, in which the 41L and 215D mutations appeared directly at seroconversion, strongly pointing at the transmissibility of the 215D virus.

When antiviral therapy is applied, the environment changes once yet again, and under conditions of suboptimal therapy in which sufficient residual viral replication can occur, a new selection for the most fit variant takes place. For patient 4, a single mutation (GAC or TCC to TAC) within the codon of amino acid 215 of RT appeared sufficient to confer a decisive replicative advantage in the presence of d4T and ddC. By using a newly developed real-time NASBA with a high throughput and dynamic quantification range, *in vitro* competitions with increasing concentrations of AZT or d4T showed that the change from 215D to 215Y by a single nucleotide mutation conferred the replicative advantage. Apparently, the AZT-resistant genotype of the virus of patient 4 is also advantageous in the presence of d4T. This finding shows that although the transmitted AZT-resistant viruses that have adapted to the drug-free environment closely resemble the 215T wild type in their phenotypes, their genotype retains many characteristics of the transmitted AZT-resistant 215Y virus.

Our observations confirm the high plasticity of the genome of HIV-1 and in particular of the RT gene. Transmission from a treated host to an untreated host immediately results in adaptation of the virus to its new, drug-free environment. Such adaptation becomes manifest as a fixation of a 215D or 215S mutation instead of reversion to the wild type of the pre-therapy era: 215T. These new wild types share AZT sensitivity with the old wild type, 215T. However, the new wild types are only one step away from 215Y and 215Y is therefore expected to arise rapidly upon administration of antiretroviral therapy when therapy is suboptimal. As we have observed, the newly established wild types can be transmitted. The monitoring of new wild types with D, S, or N at position 215 may thus be warranted in order to estimate the threat to long-term efficacy of a therapy regimen that includes nucleoside analogues.

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