Antiviral Resistance of Biologic HIV-2 Clones Obtained From Individuals on Nucleoside Reverse Transcriptase Inhibitor Therapy

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Objective: To study phenotypic and genotypic resistance of HIV-2 against nucleoside reverse transcriptase inhibitors (NRTI).

Methods: Biologic HIV-2 clones were generated from 3 patients before and after initiation of antiretroviral therapy with zidovudine (AZT) in patient RH2-7, AZT and didanosine (ddI) in patient PH2-1, and after addition of lamivudine (3TC) to AZT monotherapy in patient RH2-5. The sensitivity to NRTI of the virus clones, as defined by the 50% inhibitory concentration (IC_{50}), was determined in vitro. The predicted amino acid sequences of the reverse transcriptase proteins from these clones were determined.

Results: Comparing the sensitivity of the biologic HIV-2 clones obtained after start of therapy with those from antiviral naive patients, resistance had developed to AZT (patients RH2-7 and RH2-5) and 3TC (patient PH2-1 and RH2-5). No resistance to AZT was observed in the biologic clone from PH2-1 obtained after start of therapy. The resistant clones from RH2-5 and PH2-1, but not RH2-7, contained amino acid mutations at positions where HIV-1 has been shown to mutate after AZT and 3TC treatment.

Conclusions: Phenotypic resistance of HIV-2 to nucleoside analogues, which developed in HIV-2–infected patients treated with NRTIs, was associated with genotypic changes. Some of the mutations at amino acid positions in the HIV-2 reverse transcriptase gene corresponded with those involved in HIV-1 resistance, although no conventional mutations associated with resistance to AZT were observed.

Key Words: HIV-2—Drug resistance/resistance mutations—Antiretroviral therapy—Nucleoside reverse transcriptase inhibitors.

HIV-2, the second causative agent of AIDS, belongs, as does HIV-1, to the subfamily of Lentivirinae of the Retroviridae family (1). In vitro characteristics of both HIV-1 and HIV-2, such as tropism for $CD4^+$, α- or β-chemokine-receptor–positive cells and capacity to in-

duce cytopathic changes as well as in vivo–induced pathogenesis are similar (2–6). Despite the similarities, both perinatal and sexual transmission rates of HIV-2 are significantly lower (7–9), which has so far resulted in a more restricted spread of HIV-2 than of HIV-1. Although the virus has spread globally, it is more prevalent than HIV-1 only in certain West African countries, such as Guinea-Bissau, the Gambia, Senegal, and the Cape Verde Islands. Through trade routes, HIV-2 had limited

spread to other parts of the world, such as Angola, Mozambique, Goa, and Western Europe (10). The natural history of disease development in HIV-2 infection is protracted. Epidemiologic studies among cohorts of asymptomatic HIV-1– and HIV-2–infected individuals have demonstrated an AIDS incidence of 4.85 and 0 per 100 person years of observation in HIV-1– and HIV-2–seroincident individuals, respectively (11). These observations are in line with the immunologic changes observed in HIV-2–infected individuals. $CD4^+$ T-cell counts and CD4:CD8 ratios decline more slowly than in HIV-1–infected individuals (11). Furthermore, we have recently demonstrated significantly lower tissue and plasma viral loads in HIV-2–infected individuals than in HIV-1–infected individuals at comparable stages of disease development (submitted for publication).

In HIV-1 infection, it has been shown that both the risk of developing disease and the lack of efficacy of antiviral therapy are strongly associated with HIV-RNA plasma levels (12,13). Nucleoside reverse transcriptase inhibitors (NRTI) treatment strategy from 1987 to 1994 was initially limited to zidovudine (AZT) monotherapy, which delays disease progression in asymptomatic and advanced HIV-1 disease and decreases plasma HIV-1 RNA by approximately 0.3 $\log_{10}$ copies/ml (14,15). Subsequent results from studies on combination NRTI therapy showed slower disease progression and an improved survival for antiviral-naive HIV-1–infected patients receiving combination therapy versus monotherapy (16–18). A more sustained increase in $CD4^+$ cell counts and decrease in plasma HIV-1 RNA of approximately 1 $\log_{10}$ copies/ml were observed in individuals receiving dual therapy with two NRTIs. In HIV-1–infected patients, it has been shown that the virostatic properties of AZT, as well as the clinical and immunologic benefits, decrease during the course of monotherapy (19). Increasing evidence indicates that treatment failure of NRTI therapy is closely linked to levels of phenotypic resistance and an associated genotypic mutation pattern (20). Viral genotyping may therefore be an important tool in choosing anti-HIV therapy regimens (21–23).

Limited data on antiretroviral treatment of HIV-2 infected individuals are available. Only small differences have been observed between in vitro sensitivity of primary HIV-1 and HIV-2 isolates to AZT, didanosine (ddI) and lamivudine (3TC) (24). No data are yet available on the development of genotypic or phenotypic resistance of HIV-2 strains from patients on NRTI therapy. We therefore studied biologic clones from 3 HIV-2–infected patients treated with NRTIs and determined their phenotypic and genotypic resistance patterns.

MATERIAL AND METHODS

Patients

Patients in this study regularly visited our outpatient clinics in Rotterdam ($n = 2$) and Paris ($n = 1$). Clinical, immunologic, and virologic parameters were monitored from 1994 onward. Patient RH2-7 (a 39-year-old heterosexual man) and patient RH2-5 (a 40-year-old heterosexual woman), both living in Rotterdam, had originated from the Cape Verde Islands. Patient PH2-1 was a French-born homosexual man living in Paris. The indication for initiation or adjustment of antiretroviral therapy in all 3 patients was a low $CD4^+$ cell count. They were asymptomatic at the time of starting therapy. Relevant clinical data on antiretroviral treatment and $CD4^+$ cell counts after start of therapy are given in Figure 1. After 20 months of treatment with AZT, patient RH2-7 developed renal failure caused by HIV-nephropathy, a few months later he was diagnosed with disseminated *Mycobacterium avium* complex infection. Patient PH2-1, who had remained asymptomatic during treatment with AZT/ddI, was switched to therapy containing a protease inhibitor because of a declining CD4 cell count. Patient RH2-5 was treated with AZT monotherapy and in addition AZT/3TC combination therapy for 14 and 18 months, respectively, until she progressed to AIDS as she developed *M. tuberculosis*.

Isolation of Biologic Virus Clones

The isolation of biologic HIV clones have been described previously (6). Briefly, participant peripheral blood mononuclear cells (PBMCs) were cocultivated at three different dilutions, 2×10^2, 2×10^3, and 2×10^4 cells/well, with 3 days mitogen-prestimulated (PHA-L, Boehringer Mannheim, Germany), HIV-negative donor PBMCs (4×10^4 cells/well) in 96-well microtiter plates (Costar; Corning, NY, U.S.A.). Every week 50 µl of culture supernatant was collected from each well for detection of p24 antigen (V5 enzyme-linked immunosorbent assay [ELISA], Organon, Boxtel, the Netherlands). At the same time, 2×10^2 cells from each well were transferred to a new well, and 4×10^4 fresh mitogen-prestimulated healthy donor PBMCs were added to propagate the culture. Productively infected wells were considered to contain a clonal virus strain, when <5 of 96 wells produced positive test results. The biologic clones were further cultured using mitogen-stimulated PBMCs and expanded to high titered stocks by a 1-week passage on U87/CCR-5 cells (6). For comparison, a previously described HIV-1 molecular clone, obtained from an antiviral-naive individual was used (25,26).

Antiviral Drug Susceptibility Testing

The sensitivity of the biologic clones to NRTIs was determined in vitro, using a similar method previously described for HIV-1 drug testing (Table 1) (27). Virus stocks were thawed and titered by reverse transcriptase (RT) activity. Mitogen-stimulated CD8-depleted PBMC from HIV-seronegative donors were inoculated in triplicate with $TCID_{50}$ in the presence of 10-fold dilutions of AZT, 3TC, and ddI, starting at 10 µM. After 1 week, supernatants were tested for RT activity (28). The concentration of the drug that resulted in a 50% reduction in RT activity, the 50% inhibitory concentration (IC_{50}) was calculated by the median effect equation (27). Virus clones were considered resistant, when the IC_{50} was >5 times higher than the IC_{50} of the naive clones (in patients RH2-7 and PH2-1) or the clone before therapy adjustment (in patient RH2-5).
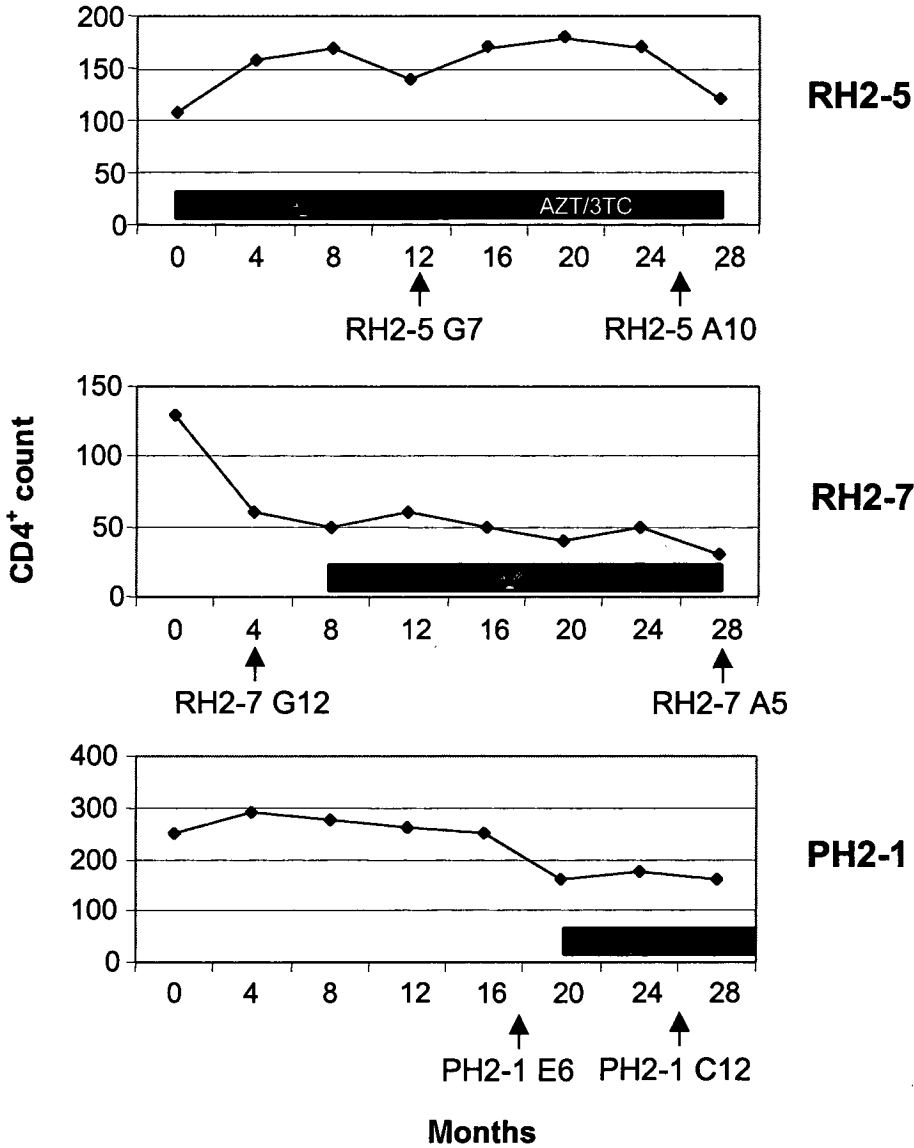
FIG. 1. CD4$^+$ cell counts in 3 HIV-2–seropositive patients treated with nucleoside reverse transcriptase inhibitors.

Polymerase Chain Reaction Detection of RT Sequences

High molecular-weight DNA was extracted with Celite beads (Janssen Chimika, Beersen, Belgium) from PBMC that were infected with the biologic virus clones from the patients (29). Amplification was performed as previously described (30). Briefly, RT sequences were amplified in a polymerase chain reaction with primers 5'RD1 (5'CCC CAA TCA ACA TTT TTG GCA G3') and 3'RD3 (3'CCT AGT TCT GCA TTT A C TTG CCC3'; kindly provided by C. Boucher) for 2 minutes at 92°C, 1 minute at 50°C, 2 minutes at 72°C for 35 cycles, with a final extension of 10 minutes at 72°C. The amplified fragments were cloned into the PCR2.1 cloning vector (Invitrogen, San Diego, CA, U.S.A.) according to the manufacturer's protocol. Clones were sequenced with the *Taq* Dye Deoxy Terminator sequencing kit on the 373A sequencing system of Applied Biosystems (Foster City, CA, U.S.A.). All clones were sequenced on both strands using custom-made primers. Alignment of the protein sequences was performed with the Lasergene software (DNAstar, Madison, WI, U.S.A.).

RESULTS

Virus Phenotypic Sensitivity to Nucleoside Reverse Transcriptase Inhibitors

Biologic HIV-2 clones were obtained from patients RH2-7 and PH2-1 before treatment (RH2-7 G12 and PH2-1 E6) and after 20 and 6 months after initiation of antiretroviral therapy respectively (RH2-7 A5 and PH2-1 C12). The first virus clone from patient RH2-5 (RH2-5 G7) was obtained after 6 months of AZT monotherapy and the second clone 12 months after adding 3TC to the

TABLE 1. *Susceptibility of HIV-2 clones to nucleoside reverse transcriptase inhibitors (NRTI)*

Virus clones	Patient drug regimen	AZT	3TC	ddI
RH2-5 G7	AZT	0.1	0.02	NT
RH2-5 A10	AZT + 3TC	0.06	0.9	NT
PH2-1 E6	None	0.01	0.02	6
PH2-1 C12	AZT + ddI	0.015	0.25	6
RH2-7 G12	None	0.005	NT	3
RH2-7 A5	AZT	0.1	NT	2
HIV-1 ACH320.2A.1.2	None	0.003	NT	0.3

For the HIV-2 biologic clones, the 50% inhibitory concentration of the NRTIs used in this study are given in micromolars.

AZT, zidovudine (azidothymidine); 3TC, lamivudine; ddI, didonosine; NT, not tested.

regimen (RH2-5 A10; Fig. 1). Sensitivity to AZT of RH2-7 G12 and PH2-1 E6 obtained from antiviral-naive patients proved to be comparable with the HIV-1 molecular clone obtained from an antiviral-naive HIV-1–seropositive patient. The ddI concentration required to inhibit replication of all HIV-2 clones and the HIV-1 clone was high and therefore did not allow us to determine development of resistance to ddI. This findings, also observed by others, may be caused by the inefficient conversion of ddI to the biologically active form dideoxyadenosine (ddA)-triphosphate when activated PBMCs are used for phenotype resistance (31,32).

Sensitivity of the biologic HIV-2 clones obtained after start of therapy were compared with those obtained before therapy or, for the clones obtained from patient RH2-5, to clones from antiviral-naive individuals. The biologic clones from patients on AZT monotherapy (patients RH2-5 and RH2-7) were 10 to 20 times less sensitive to inhibition by AZT. Sensitivity to AZT RH2-5 A10 relative to RH2-5 G7 remained unchanged. No resistance to AZT was observed for PH2-1 C12. Both clones from patients on dual NRTI therapy were less sensitive to inhibition with 3TC, clone PH2-1 C12 was 10 times less sensitive and clone RH2-5 A10 45 times less sensitive.

Genotypic Analysis of Biologic Clones

The RT genes from the biologic HIV-2 clones were sequenced (Fig. 2). From several biologic clones, this sequence was determined directly on the PCR product. In none of these, however, were multiple polymorphisms observed, thus supporting the clonality of these biologic clones. Multiple differences in amino acid sequences were observed between the clones obtained before and after therapy. For convenience, the predicted amino acid differences between the clones before and after therapy are given in Table 2.

HIV-1 RT and HIV-2 RT have similar catalytic properties and a 60% sequence identity (33). The observation that the folding of the palm of the more distantly related RT of murine leukemia virus is the same as HIV-1 RT supports the idea that HIV-1 RT and HIV-2 RT are sufficiently similar that amino acids in equivalent positions in the sequence will have similar roles in the two enzymes. Therefore, we set out to study positions within the RT gene where HIV-1 mutates when resistance due to NRTIs occurs.

The predicted amino acid sequence of RH2-7 A5 showed no mutations relative to RH2-5 G12 at positions analogous to those associated with resistance to AZT in HIV-1 infection.

Genotypic analysis of the first biologic clone from patient RH2-5, after 6 months on AZT, revealed a methionine at position 151, which may explain the relative resistance to AZT. Because we do not have virus strains from patient RH2-5 before initiation of treatment, we cannot be sure whether this mutation was indeed induced by AZT selection pressure. However, in none of the hitherto described HIV-2 virus isolates of therapy-naive patients' mutations at this position have been observed (34).

Genotypic analysis after dual therapy with NRTI was done on the clones obtained from PH2-1 (AZT + ddI) and RH2-5 (AZT + 3TC). Both virus clones showed a M184V mutation. For HIV-1, this mutation is associated with a strongly reduced sensitivity to 3TC in phenotypic assays (23). The second clone obtained from patient RH2-5 showed isoleucine instead of methionine at position 151. Furthermore, a M41I mutation appeared, which is for HIV-1 a position where mutations may occur that are associated with low level resistance to AZT (20).

DISCUSSION

In this paper, we have described phenotypic and genotypic resistance to NRTIs of biologic HIV-2 clones obtained from patients receiving treatment. Mutations associated with resistance, although in some cases occurring at similar positions as would occur in HIV-1, proved to be different with respect to topography and time of appearance when compared with mutations generally observed in HIV-1.

In HIV-1 infection, the genotypic resistance pattern for NRTI correlates with the level of phenotypic resistance (20). Loss of antiviral efficacy acquired under selection pressure of AZT monotherapy involves a gradual and stepwise accrual of mutations in functional regions of the RT gene at positions K70R, T215Y, and M41L, respectively. Although the second virus clone

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Consensus  PVAKVEPIKVTLKPGKDGPKL.QWPLTKEKIEAL.EICEKMEKEGQLEEAPPTNPYNTPTFAIKKKDKNKWRMLIDFRELNRVTQDFTEIQLGIPHPAGL
                    10        20        30        40        50        60        70        80        90       100
RH2-5  G7  ........M...........K.......................................................................
RH2-5  A10 ........M...........R.......................................................................
RH2-7  G12 ......V...T.........R.............................R...........................K..............
RH2-7  A5  ......V.............R.............................S...........................K.A.............
PH2-1  E6  ..I...............R.K.....................K....................R...........................
PH2-1  C12 ..I...............R.K.....................K....................R...........................

Consensus  AKKRRITVLDVGDAYFSIPLDEDFRQYTAFTLPSVNNAEPGKRYIYKVLPQGWKGSPAIFQ.TMRQILEPFRKAN.DVILIQYMDDLIASDRTDLEHDR
                   110       120       130       140       150       160       170       180       190       200
RH2-5  G7  .............G...............................M.......H....V........Q.I........................
RH2-5  A10 .............G...............................I.......H....V........Q..I.......................
RH2-7  G12 P.......C.........................................W............S...............................
RH2-7  A5  P.......C.........................................W............S...............................
PH2-1  E6  ...K...........K...........................................F.....P..........G..K..............
PH2-1  C12 ...K...........K...........................................F.....P..........G..K..............

Consensus  VVLQLKELLNGLGFSTPDEKFQ.DPPYQWMGYELWP.KWKLQKIQLPQKEIWTVNDIQKLVGVLNWAAQIYPGIKTRHLCRLIRGKMTLTEEVQWTELAE
                   210       220       230       240       250       260       270       280       290       300
RH2-5  G7  .........................R.........T...............................V.........................
RH2-5  A10 ...............R.........R.........N...............................V...........H..............
RH2-7  G12 .........................R.........N.........................................R................
RH2-7  A5  .........................K.........N...V.......................................L.............
PH2-1  E6  .........................K..F......T...L.....K..............................................
PH2-1  C12 .........................K..F......T...L...R........................K.........G..............

Consensus  AELEENRIILSQEQEGHYYQEEKELEATVLKDQDNQWTYKIHQEEKILKVG
                   310       320       330       340       350
RH2-5  G7  .......................E..........G................
RH2-5  A10 .......................E..........G.......L........
RH2-7  G12 ...K...........G.................................
RH2-7  A5  ...K...........G.................................
PH2-1  E6  ........Y..............I..SE.....................
PH2-1  C12 ........C..............I..SE.....................
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FIG. 2. Alignment of the predicted amino acid sequence from HIV-2 reverse transcriptase protein fragments. The arrows indicate mutations corresponding with nucleoside reverse transcriptase inhibitor resistance in HIV-1.

TABLE 2. *Changes of amino acids on the RT gene obtained from 3 HIV-2 clones from patients after therapy with nucleoside reverse transcriptase inhibitors*

RH2-7 A5	RH2-5 A10	PH2-1 C12
T14P (ACA→CCA)	K22R (AAA→AGA)	E122K (GAA→AAA)
P51S (CCT→TCT)	K35S (AAA→AGT)	K125R (AAG→AGG)
T84A (ACT→GCT)	**M41I (ATG→ATA)**	**M184V (ATG→GTG)**
R223K (AGA→AAA)	E49R (GAA→AGA)	Q248R (CAG→CGG)
E250D (GAG→GAT)	V111I (GTA→ATA)	K277R (AAA→AGA)
R286K (AGA→AAA)	**Q151I (CAG→ATA)**	R281K (AGA→AAA)
M287L (ATG→TTG)	**M184V (ATG→GTG)**	E292G (GAA→GGA)
	G213R (GGA→AGA)	Y317C (TAC→TGC)
	T237N (ACC→AAC)	
	V350L (GTA→TTA)	

Mutations at positions that are associated with HIV-1 resistance to NRTI are printed in bold.

from patient RH2-7, which was obtained after 20 months of AZT monotherapy, was 20-fold less sensitive to AZT in the phenotypic assay, genotypic analysis did not show any mutations associated with resistance to AZT. To further elucidate this discrepancy, an additional biologic clone, taken from the same patient at the same timepoint, was sequenced. Moreover, in this biologic clone no mutations were found at positions associated with HIV-1 AZT resistance (data not shown). Site-directed mutagenesis should be done to be able to define regions and positions that are involved in resistance to zidovudine HIV-2 RT. In patient RH2-5, the virus clone obtained after 12 months of AZT monotherapy was phenotypically resistant to AZT. Genotypic analysis showed the appearance of methionine at position 151. The Q151M mutation requires a change of two nucleotides within one codon. In HIV-1 infections, this mutation develops in asymptomatic individuals after more than 1 year of combination therapy with AZT and zalcitabine (ddC) or ddI (35,36). This mutation has been shown to induce resistance to multiple NRTIs in HIV-1 infection and is associated with a set of other mutations, including A62V, V75I, F77L, and F116Y (35,36). Among these five mutations, Q151M is thought to be the first mutation to develop. Until now those factors that predispose virus strains to develop the set of multidrug resistance mutations in favor of AZT resistance mutations are not known. It has been suggested that certain nucleotide or amino acid sequences predispose these viruses to avoid any AZT-related mutations (36). In virus clone RH2-5 A10, no new mutations associated with multidrug NRTI resistance in HIV-1 infection were observed.

In HIV-1 infection, the benefit of combination therapy with two NRTIs compared with monotherapy may be explained by constraints on coincidental developing resistance mutations for two NRTIs (37,38). Furthermore, it has been shown that mutations that confer resistance to one NRTI may prevent mutations associated with resis-

tance to other NRTI in the regimen (39,40). Susceptibility of the virus PH2-1 C12 to 3TC, obtained after 6 months of AZT/ddI combination therapy, was significantly lower compared with in PH2-1 E6. This decrease most probably resulted from the change of methionine to valine at position 184. This mutation was unexpected and early, because in HIV-1 infection, the M184V mutation is generally observed after 3TC or AZT/3TC therapy and rarely after AZT/ddI combination therapy. In HIV-1 infection, the M184V mutation may develop within 4 to 12 weeks after initiation of AZT/3TC treatment, whereas genotypic resistance to AZT is generally rare or absent after 24 weeks (39,40). Monotherapy with either AZT or ddI induces mutations associated with resistance to AZT or ddI within 48 weeks in most patients (37). During dual therapy with AZT and ddI, mutations associated with resistance to ddI (codon 69, 74, or 184) were rarely observed. The number of mutations associated with AZT resistance was comparable with the number found in the AZT monotherapy group. It has therefore been suggested that selection for AZT resistance may impose a background that restricts evolution of ddI resistance. The genotypic resistance pattern in patient PH2-1, the M184V mutation, and the absence of AZT resistance seem to be more in congruent with AZT/3TC therapy in HIV-1–infected individuals than to AZT/ddI therapy. It may be hypothesized that for optimal enzymatic activity of HIV-2 RT the M184V mutation is preferable.

In conclusion, genotypic mutations in the HIV-1 RT gene are predictive for phenotypic resistance to NRTI and clinical failure and are relevant for making rational effective drug choices. In our study, we have shown that this correlation of phenotypic and genotypic resistance is less clear in HIV-2 infection. Switching NRTI therapy in HIV-2–infected individuals cannot be decided only on genotypic analysis results. As the number of HIV-2–infected patients receiving antiretroviral treatment increases, further studies are needed.

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