Mutations in the pol Gene of Human Immunodeficiency Virus Type 1 in Infected Patients Receiving Didanosine and Hydroxyurea Combination Therapy

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The pattern of mutations in the reverse transcriptase (RT) of human immunodeficiency virus type 1 (HIV-1) strains that confer resistance to didanosine (ddI) was analyzed in 2 groups of patients receiving either ddI monotherapy or ddI plus hydroxyurea (HU) combination therapy. Twelve patients receiving combination therapy and 8 receiving monotherapy were tested. Combinations of ddI plus HU did not prevent the onset of mutations, which emerged in 50% of the patients in this group compared with 25% of the ddI monotherapy group. In addition, in 1 patient from the combination therapy arm, who had a limited response to the therapy, an unusual pattern of mutations was found: the insertion of 2 amino acids between residues 69 and 70, a region critical for resistance to nucleoside analogs. The higher efficacy of the combination of HU and ddI compared with that of ddI monotherapy cannot be attributed to a delayed or decreased onset of resistance to ddI.

Drug-resistant mutants of human immunodeficiency virus type 1 (HIV-1) emerge under the selective pressure of prolonged chemotherapy. There are specific mutations in the HIV-1 reverse transcriptase (RT) gene that confer resistance to the nucleoside analogues, such as zidovudine, didanosine (ddI), zalcitabine (ddC), and lamivudine [1]. Mutation at codons 41, 67, 70, 215, and 219 of HIV-1 RT confer resistance to zidovudine [1, 2]. HIV-1 strains with mutations at codons, 62, 75, 77, 116, and 151 have been reported in patients receiving zidovudine and ddI combination therapy; these mutations confer on the virus a reduced sensitivity to multiple antiretroviral dideoxynucleosides, and 151 is a critical mutation in the development of multidrug resistance [3, 4]. Although mutation at codon 74 is considered to be the primary mutation responsible for ddI resistance [5–7], other mutations have been reported that decrease the susceptibility of HIV-1 to ddI. These mutations are located at codons 65 and 184 [8, 9].

Combinations of drugs have been used to limit negative effects due to the emergence of mutations. Interest has grown in the use of hydroxyurea (HU) and ddI as anti–HIV-1 therapy [10–18]. We have been studying the in vitro and in vivo effects of ddI plus HU therapy [10, 11, 14, 19]. We compared, after 24 weeks of treatment, the genotypic changes in the HIV-1 RT gene associated with ddI resistance in ddI-treated patients who did or did not receive HU.

Methods

Patients. Twenty patients were selected from a larger group of 58 who were in an ongoing clinical trial. To be eligible, each patient needed documentation of HIV-1 infection by ELISA confirmed by Western blot. Upon entry, each patient was required to have 250–500 CD4 cells/mL determined one time within 2 weeks before enrollment. Patients were randomly assigned to each group; randomization was done by use of a computer-generated two-digit numeric list. On the basis of the second digit, patients were enrolled into a particular study arm.

Blood samples were obtained prior to therapy and again after 24 weeks of therapy from 20 patients treated with either the combination of ddI plus HU or ddI monotherapy. Twelve of these patients were treated with ddI plus HU (group I), and 8 were treated with ddI monotherapy (group II). Eight patients enrolled in group I and 5 enrolled in group II had received zidovudine therapy for >1 year prior to our study, but they stopped this therapy at least 60 days before the beginning of this clinical trial.

Patients treated with ddI monotherapy received ddI at the following concentrations twice daily, according to weight: Patients weighing <50 kg were given 200 mg; those weighing 50–75 kg were given 400 mg; and those weighing >75 kg were given 600 mg. Patients treated with ddI plus HU combination therapy received 500 mg of HU plus ddI in the same doses as indicated above, twice daily.

Plasma viremia. Plasma viremia was determined by using a commercially available RT–polymerase chain reaction (PCR) kit with a detection limit of 200 copies/mL HIV-1 RNA (Roche Diagnostic Systems, Branchburg, NJ). Patients analyzed by sequencing were selected based on the plasma viremia value obtained before therapy and after 24 weeks of treatment. This analysis included patients with a plasma viremia of >800 copies/mL.

RT-PCR. Viral RNA was isolated from 200 μL of plasma by using a TRIzol reagent (Life Technologies GIBCO BRL, Gaithers-
burg, MD) according to the manufacturer’s protocol. cDNA was obtained from the RT reaction at 43°C by using the antisense primer R8 (5′-CATTTATCGGATGGTGCT-C3′, nt 3242–3264 of the HXB2 sequence) [20]. The reaction conditions were as follows: 0.4 μM primer, 400 μM each nucleoside triphosphate, 10 mM dithiothreitol, 4 mM MgCl2, 20 mM TRIS-HCl (pH 8.4), 50 mM KCl, 200 U of SuperScriptII RT (Life Technologies), and 10 U of RNase inhibitor in a final volume of 25 μl. A fragment of 765 bp encompassing codons 1–238 of RT was amplified by using the sense primer F1 (5′-GGACCTACCTGCTCACAT-3′, nt 2483–2502 of the HXB2 sequence) and the antisense-primer R12 (5′-TTGCATACCCCATCAAGG-3′, nt 3230–3247 of the HXB2 sequence). The PCR reaction mixture contained the following: 3 μl of first-strand cDNA, 0.2 μM each primer, 100 μM each nucleoside triphosphate, 1.5 mM MgCl2, 20 mM TRIS-HCl (pH 8.4), 50 mM KCl, and 5 U of Taq DNA polymerase (Boehringer Mannheim, Indianapolis) in a final volume of 100 μl. The cycle conditions were 95°C for 3 min, 50 times (94°C for 20 s, 58°C for 25 s, and 72°C for 30 s), and 72°C for 10 min.

If the PCR products were not visible on an agarose gel using ethidium bromide staining, a second seminested PCR was performed using the sense primer F5 (5′-CGAGCATGGATGCTCGGCAAAAGT-3′, nt 2589–2610 of the HXB2 sequence) and the antisense-primer R12, amplifying a fragment of 659 bp (encompassing codons 15–233 of RT). Primers F1 and F5 have been previously described [21, 22]. The nested PCR conditions were 1 μl from the first PCR reaction, 0.4 μM each primer, 200 μM each nucleoside triphosphate, 1.5 mM MgCl2, 10 mM Tris-HCl, 50 mM KCl, and 1 U of Taq DNA polymerase (Boehringer Mannheim) in a final volume of 50 μl. The cycle conditions were 95°C for 3 min, 45 times (94°C for 20 s, 55°C for 20 s, and 72°C for 20 s), and 72°C for 10 min.

**Sequencing.** The PCR products were treated with shrimp alkaline phosphatase and Escherichia coli exonuclease I (Amersham Laboratories, Arlington Heights, IL) to remove the residual PCR primers and dNTPs (for details see [23, 24]). Following that, cycle sequencing with Dye Terminator and the AmpliTaq FS enzyme (Perkin-Elmer ABI) was performed. Three primers were used for sequencing: the sense primer F9 (5′-GGCATGACAGAGAAGAAA-3′, nt 2619–2638 of the HXB2 sequence), the sense primer F7 (5′-GGATGGAAAGGATCACCGC-3′, nt 3002–3021 of the HXB2 sequence), and the antisense primer R6 (5′-TACTAGTGTTAGTAAATGCGT, nt 2930–2951 of the HXB2 sequence). The sequencing reactions were performed according to the manufacturer’s protocol (Perkin-Elmer ABI) and were run in both the automatic ABI 373 and ABI 377 sequencers. The analysis and assembling of sequence data was performed with EditView 1.0 (Perkin-Elmer ABI) and SeqMan 3.50d20 (DNASTAR, Madison, WI).

For each patient, the sequences were determined on both DNA strands, using PCR products amplified at a different time. To minimize the possibility of errors, the RNA extraction and the RT-PCR and the sequencing reactions were repeated twice on most of the samples.

**Results**

**Plasma viremia.** The patient population described here is part of a larger trial (Lori F et al., unpublished data). In the larger population, in 38 patients treated with ddI plus HU, a decrease of plasma viremia of 1.32 log was shown at week 24. In contrast, in 20 patients treated with ddI, the decrease of viremia was 0.77 log. This difference was statistically significant (P < .001). In the present study, only patients with >800 viral copies/mL after 24 weeks of treatment were included because viral DNA could not be amplified and sequenced with fewer than that number of viral copies. Accordingly, we selected a group of patients (12 patients from the combination therapy arm and 8 from the monotherapy arm) who were not fully representative of the larger population.

**Sequence analysis.** The sequences containing a 618-bp fragment of the HIV-1 RT gene (from residue 21 to residue 227 of HIV-1 RT) were obtained before therapy and after 24 weeks of therapy. We searched for mutations after sequences were aligned in a single contig (the alignment of homologue sequences). Special attention was given to those mutations previously recognized to confer resistance to ddC (codons 65, 74, and 184) [5, 8, 9].

After 24 weeks of therapy, 6 of 12 patients from group I and 2 of 8 patients from group II acquired mutations in positions that have been described to confer resistance to ddI.

In group I, mutations appeared mainly in the area of residues 69–74 (figure 1). Six patients developed the L74V mutation, and in 1 of these 6 patients, a M184V mutation was also observed. Another patient developed a T69S mutation in the same position as the T69D mutation, which is known to confer resistance to ddC [25]. That same patient also acquired a previously not described insertion; 6 nucleotides (AGTGGT) were inserted between residues 69 and 70, resulting in an in-frame serine-glycine insertion.

In group II, 1 patient developed a K65R mutation, and a second developed the L74V mutation. Other less relevant mutations were found: at position 135 (patient 14), possibly because of the highly polymorphic nature of this region [26], and at position 69 (patient 17). This T69A mutation is in the same position as the T69D mutation, known to confer resistance to ddC [25]. A mixed population of wild type and emerging mutants was found in patients 1, 8, 14, and 17 (figure 1, parentheses). Analysis of the HIV-1 RT sequences from patients before starting therapy (baseline) confirmed that there were no ddI-resistant mutations prior to therapy.

Eight of the 13 patients who previously received zidovudine therapy also had mutations in the HIV-1 RT gene known to confer resistance to zidovudine [2]. Six of them were enrolled in group 1 and 2 in group II. No significant changes in these mutations occurred between the time patients started ddI or ddI plus HU therapy and 24 weeks after this therapy was started. However, patient 6 lost the D67N mutation between these two time points and gained a K70R mutation.

Patient 6 responded less to the therapy than did all other patients in group I (figure 2A). Sequences of the viral RT gene were obtained from this patient at different time points before therapy and at weeks 8, 12, and 24. After 12 weeks of therapy,
Figure 1. Changes of amino acid sequence in HIV-1 reverse transcriptase at week 24 of therapy. ddI designates positions known to be selected for didanosine (ddI)-resistant mutations; zidovudine (AZT) designates positions known to be selected for AZT-resistant mutations. # indicates patients who received AZT previously. * indicates that no sequence was available at baseline. ** indicates amino acids also present at baseline. Relevant mutations are in bold. Mixed populations of wild type and mutants are in parentheses.

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Figure 1. Changes of amino acid sequence in HIV-1 reverse transcriptase at week 24 of therapy. ddI designates positions known to be selected for didanosine (ddI)-resistant mutations; zidovudine (AZT) designates positions known to be selected for AZT-resistant mutations. # indicates patients who received AZT previously. * indicates that no sequence was available at baseline. ** indicates amino acids also present at baseline. Relevant mutations are in bold. Mixed populations of wild type and mutants are in parentheses.

A T69S mutation appeared, together with an insertion of 2 amino acids (Ser-Ser) between residues 69 and 70. After 24 weeks, a further change was observed: The second serine (of the insertion) changed to glycine, resulting in a Ser-Gly insertion (figure 2B). We can exclude the possibility that this was due to contamination because several RT-PCR reactions were done at different times, starting from independent RNA preparations. The sequence data of the RT-PCR products on both strands were consistent.

**Discussion**

RT is made of two subunits, p66 (560 amino acids) and p51 (440 amino acids). In the polymerase domain, there are four subdomains, called fingers, palm, thumb, and connection. The polymerase active site is located in the palm subdomain of p66. It is known that nucleoside analogues bind to a hydrophobic pocket close to the active site in the p66 subdomain [27]. Most of the mutations that confer resistance to the nucleoside inhibitors do not lie in the immediate vicinity of the polymerase active site but are located within both the finger and palm subdomains, in a region that interacts with the template primer (the region spanning residues 65–74) [28, 29]. It was suggested that the finger subdomain of p66 could be involved in the appropriate positioning and conformation of the template strand that could influence the polymerase active site. Therefore, mutations in that region could indirectly influence the ability of the active site to accept or reject the incoming nucleotides [28].

The majority of the mutations we found were concentrated due to contamination because several RT-PCR reactions were done at different times, starting from independent RNA preparations. The sequence data of the RT-PCR products on both strands were consistent. Most of the mutations that confer resistance to the nucleoside inhibitors do not lie in the immediate vicinity of the polymerase active site but are located within both the finger and palm subdomains, in a region that interacts with the template primer (the region spanning residues 65–74) [28, 29]. It was suggested that the finger subdomain of p66 could be involved in the appropriate positioning and conformation of the template strand that could influence the polymerase active site. Therefore, mutations in that region could indirectly influence the ability of the active site to accept or reject the incoming nucleotides [28].

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Although this patient responded to the combination of HU plus ddI, his response was the least successful. This confirms that the region between 65 and 74 is critically associated with changes in the sequence of HIV-1 RT after treatment with ddI.

Ten of 12 patients in the combination therapy arm and 6 of 8 in the monotherapy arm were also screened for the presence...
Figure 2. Identification of a novel HIV-1 reverse transcriptase (RT) mutant in a patient (no. 6) treated with ddI plus hydroxyurea combination therapy. A. Plasma viremia during 24 weeks of treatment. B. Sequence of HIV-1 RT gene between residues 64 and 75. In the nucleotide sequence R = G or A. Nos. shown represent amino acid positions.

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References


