High-Level Resistance to 3'-Azido-3'-Deoxythimididine due to a Deletion in the Reverse Transcriptase Gene of Human Immunodeficiency Virus Type 1

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A variant of human immunodeficiency virus type 1 (HIV-1) possessing a deletion in the reverse transcriptase (RT) gene at codon 67 was identified in a patient who had failed combination antiretroviral therapy. This deletion initially emerged under the selective pressure of combination therapy with 3'-azido-3'-deoxythimidine (AZT) plus 2',3'-dideoxyinosine. It has persisted for more than 3 years in association with the accumulation of a variety of other well-described drug resistance mutations and an uncharacterized mutation at RT codon 69 (T69G). Phenotypic studies demonstrated that the codon 67 deletion by itself had little effect on AZT sensitivity. However, in the context of the T69G mutation and three other mutations known to be associated with AZT resistance (K70R, T215F, and K219Q), this deletion led to a increase in AZT resistance from 8.5-fold to 445-fold. A further increase in resistance (up to 1,813-fold) was observed when two mutations associated with nonnucleoside RT inhibitor resistance (K103N and L74I) were added to the deletion T69G K70R T215F K219Q construct. Hence, these results establish that a deletion at RT codon 67 may be selected for in the presence of antiretroviral therapy and may lead to high-level resistance to AZT.

Current studies of the treatment of patients with human immunodeficiency virus type 1 (HIV-1) infection with combinations of antiretroviral drugs have demonstrated a dramatic reduction in AIDS-related morbidity and mortality (11, 20, 36, 39). These therapies, while potent, are not capable of eradicating HIV-1, and thus, today, the medical management of patients with HIV-1 infection requires the development of long-term strategies (6, 14, 38, 51). Treatment failure is a complex phenomenon (41). It is typically characterized as an inability to achieve adequate suppression of viral replication and may be due to the emergence of drug-resistant virus and/or noncompliance with the prescribed medical regimen (13, 49). Genotypic analyses have revealed that drug-resistant viruses may acquire mutations not only in protease (PRT) and reverse transcriptase (RT) coding regions, but also in the gag-pol cleavage sites (53). Once multidrug resistance viruses emerge in the setting of combination therapy, it is often difficult to regain control of viremia (41, 49). Thus, studies of the precise genetic features of drug resistance and viral fitness are of value in achieving a better understanding of these phenomena and in developing better salvage regimens.

Long-term treatment with 3'-azido-3'-deoxythimidine (AZT) can result in the development of AZT resistance (27, 34, 43). Amino acid substitutions in the HIV-1 RT at amino acid codons 41, 67, 70, 210, 215, and 219 have all been well characterized as conferring AZT resistance (19, 21, 24, 28). To acquire high-level resistance (50% inhibitory concentrations [IC50s] increased more than 100-fold) to AZT, the accumulation of four to six mutations in the RT gene is generally required (24, 30, 44). In contrast to AZT resistance, high-level resistance to nonnucleoside RT inhibitors (NNRTIs) is generally conferred by a single mutation in HIV RT at either codon 103 (N to K) or codon 181 (Y to C) (44).

Patient profile. In the present study, we have identified a patient with persistent high levels of viral replication despite multiple regimens of combination antiviral chemotherapy (Fig. 1). The patient is a 52-year-old man who was initially enrolled in a monotherapy study of an NNRTI (L697,661) (16). He was subsequently treated with AZT monotherapy for 10 months, followed by AZT plus didanosine (ddI) combination therapy for 22 months. He then received AZT plus ddI plus delavirdine (10) for the next 4 months, followed by a switch to indinavir (IND) (48) monotherapy and subsequently a series of combination regimens, including zalcitabine (ddC), lamivudine (3TC) (46), stavudine (d4T) (32), abacavir (7), ritonavir (33), saquinavir (42), amprenavir (E. E. Kim, B. G. Rao, D. D. Deininger, C. T. Baker, M. D. Dwyer, M. A. Navia, T. A. Thomon, and R. D. Tung, Abstr. 10th Int. Conf. AIDS, abstr. 319A, 1994), efavirenz (52), and hydroxyurea (3). Over this 7-year period, he also received 28 cycles of intermittent interleukin 2 (IL-2) therapy. A detailed description of these therapies and the HIV-1 RNA and total CD4+ cell counts during this time are shown in Fig. 1. Particle-associated HIV-1 RNA levels in plasma were determined by the branched-DNA (bDNA) signal amplification assay (Chiron) (9). The detection limit of the assay before September 1996 (version 1) was 10,000 copies per ml. From September 1996 to August 1998, the detection limit of the assay (version 2) was 500 copies/ml. After August 1998, the detection limit of the assay (version 3) was 50 copies/ml. As can be seen, this is a patient for whom sustained virologic control has not been possible.

To attempt to elucidate the mechanisms leading to multidrug resistance in this patient, nucleic acid analysis was performed with a 1.4-kb fragment of the gag, PRT, and RT region of the HIV-1 genome (nucleotides 2010 to 3492) by using a...
plasma sample from October 1997. PCR amplifications were performed as previously described (53). A total of 10 clones were sequenced. Changes in the gag, PRT, and RT regions were compared with the HIV-1 clade B consensus sequence as a reference (37). Multiple mutations were observed in RT (Table 1). Mutations associated with resistance to AZT (M41L, K70R, L215F, and K219Q), 3TC (M184V), and NNRTIs (K103N, A98G, L74I, Y188C, and P236M) were detected (44). In addition, a deletion was observed at RT codon 67 (D67). Novel amino acid substitution patterns were observed at codons 69 (T69G), 75 (V75T), and 179 (V179I). Other mutations were seen at codons known to be associated with ddC (T69D) or NNRTI (V75L/I and V179D/E) resistance (15, 25, 44). No amino acid substitutions were observed at the codons associated with multinucleoside analog resistance (codons A62, F77, F116, and Q151) (45). In addition, position P2 in the p7gag–p10gag cleavage site was mutated from A to V (53). The PRT sequence revealed that there were nine amino acid substitutions (L10I, M36I, M46I, I54V, L63P, A71V, G73S, V82A, and L90M) (44).

To determine when the codon 67 deletion arose and if it emerged in a sequential manner along with resistance mutations, analyses were performed with longitudinal samples spanning 6 years (Table 1). Due to the limited availability of plasma samples from the earlier time points, proviral DNA was used as the primary source for sequence information. In December 1991 (initiation of AZT therapy), all 10 clones were wild type. By January 1993 (3 months following the addition of ddI and the time of maximal virus suppression), multiple mutations were noted in the RT gene, including a D67N substitution. One year following this, while the patient was still on AZT plus ddI, the Δ67 deletion was first seen. This unusual deletion was

**TABLE 1. Emergence of HIV-1 variants containing mutations in the RT gene during combination therapy**

<table>
<thead>
<tr>
<th>Time point</th>
<th>Virus</th>
<th>M41</th>
<th>D67</th>
<th>T69</th>
<th>L74</th>
<th>V75</th>
<th>A98</th>
<th>K100</th>
<th>K103</th>
<th>V179</th>
<th>M184</th>
<th>Y188</th>
<th>L210</th>
<th>K219</th>
<th>P236</th>
</tr>
</thead>
<tbody>
<tr>
<td>December 1991</td>
<td>P</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>January 1993</td>
<td>P</td>
<td>N/-</td>
<td>R/-</td>
<td></td>
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</tr>
<tr>
<td>January 1994</td>
<td>P</td>
<td>N/Δ</td>
<td>R/-</td>
<td>I/-</td>
<td></td>
<td></td>
<td></td>
<td>N/Δ-</td>
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</tr>
<tr>
<td>December 1994</td>
<td>P</td>
<td>Δ/-</td>
<td>G/-</td>
<td>R/-</td>
<td>I/-</td>
<td></td>
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</tr>
<tr>
<td>October 1997</td>
<td>P</td>
<td>Δ</td>
<td>G</td>
<td>R</td>
<td>I/-</td>
<td>T/-</td>
<td>G/-</td>
<td>I/-</td>
<td>N/-</td>
<td>V</td>
<td></td>
<td>C/-</td>
<td>F</td>
<td>Q</td>
<td>-/M</td>
</tr>
<tr>
<td>December 1994</td>
<td>B</td>
<td>Δ</td>
<td>G</td>
<td>R</td>
<td>I</td>
<td>T</td>
<td>G</td>
<td>I/-</td>
<td>N/-</td>
<td>V</td>
<td></td>
<td>C/-</td>
<td>F</td>
<td>Q</td>
<td>-/M</td>
</tr>
</tbody>
</table>

* Mutations in the RT gene were identified at seven different time points, which correspond to the times indicated by arrows in Fig. 1. All data were obtained from 8 to 10 clones at each time point by direct amplification of samples.
* P and B, proviral DNA and blood virus (plasma virus), respectively.
* Major mutations are listed first, followed by slashes (/) and minor mutations. Dashes (—) denote identity with the HIV-1 consensus B sequence.
found in 8 of 10 clones from 1994 and 10 of 10 clones from 1997. The T69G substitution emerged at the same time as Δ67. Although the T69G change was seen in clones in which codon 67 was present, the Δ67 deletion was only seen in clones that contained the T69G mutation (Table 1). A recent abstract also reported the emergence of a deletion at codon 67 in association with a T69G change during combination therapy with 3TC, D4T, and IN (L. Ross, M. Johnson, N. Graham, M. Shaefer, M. Griswold, and M. St. Clair, Abstr. 5th Conf. Retroinfec., abstr. 679, 1998). The emergence of mutant forms of RT with two to three amino acid inserts between RT codons 69 and 70 have also been reported (8, 50; Ross et al., Abstr. 5th Conf. Retroinfec.). These residues are all part of the finger domain of RT (22, 47). Site-directed mutagenesis studies confirmed the important role of this insert in conferring reduced susceptibility to ddI, ddC, 3TC, and 9-(2-phosphonylmethoxyethyl)adenine (PMEA) (40), but not to AZT or d4T. This two-codon insert plus a T69S mutation in the context of AZT resistance mutations decreased susceptibility to AZT approximately 200-fold (50). Thus, a codon 69 substitution seems to be an important accompaniment of insertions or deletions in the finger domain of RT. Given the lack of prior reports (21, 31), it is likely that the prevalence of this Δ67 deletion is very rare. The crystal structure for the HIV RT was recently reported by using a combinatorial disulfide cross-linking strategy (22). That study revealed that the binding of the template-primer hybrid and the deoxyinosine triphosphate (dNTP) complexes to the RT enzyme induces a significant conformational change in the enzyme, in which there is a closure of the outer part of the finger domain toward the palm domain. Amino acids from codons 67, 69, and 74 define the B3-B4 loop in the finger domain of the HIV-1 RT (22, 47), and, therefore, one can speculate that the Δ67 deletion may influence this aspect of RT structure. The importance of this interaction is supported by the fact that many of the key mutations that enhance AZT resistance are located in this finger domain. Taken together, these observations suggest that the emergence of the codon 67 deletion may be dependent upon a prior change in the structure or function of RT.

**Drug susceptibility of chimeric HIV.** To further assess the potential relevance of this deletion at RT codon 67, a series of phenotypic analyses were carried out with recombinant viruses. A chimeric HIV was constructed with NL4.3 (1) as the parental HIV-1 strain and a cloned RT from the patient from December 1994. This chimera, HIV_R1310D, contained the deletion at codon 67; three amino acid substitutions associated with AZT resistance (K70R, T215F, and K219Q), two mutations associated with NNRTI resistance (L74I and K103N) (44), a substitution of T69G, and 11 additional amino acid substitutions of unknown significance (V35I, S48G, E102K, K122Q, I135M, C162S, G196E, R277K, R284K, V291I, and Q297A). Phenotypic studies were performed by previously described methods (2, 53) with MT-2 cell lines (17, 18).

Table 2 compares the drug susceptibility profiles of wild-type NL4.3 and the chimera HIV_R1310D to four RT inhibitors and one PRT inhibitor. The PRT inhibitor IND (48) was used as a control inhibitor for these experiments. The chimera,

### Table 2. Drug susceptibilities of chimeric HIV constructs

<table>
<thead>
<tr>
<th>Virus</th>
<th>Wild type</th>
<th>HIV_R1310D</th>
<th>HIV_R1310D+D67</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDV</td>
<td>19.8 ± 3.11 (1)</td>
<td>15.8 ± 5.77 (0.8)</td>
<td>22.4 ± 3.18 (1.2)</td>
</tr>
<tr>
<td>AZT</td>
<td>17.1 ± 6.07 (1)</td>
<td>24.800 ± 9.000 (1,450)</td>
<td>118 ± 74.8 (6.9)</td>
</tr>
<tr>
<td>ddI</td>
<td>245 ± 157 (1)</td>
<td>230 ± 70 (0.9)</td>
<td>1,967 ± 262 (6.5)</td>
</tr>
<tr>
<td>ddC</td>
<td>25.2 ± 14.8 (1)</td>
<td>87.7 ± 25.1 (3.5)</td>
<td>322 ± 43.3 (13)</td>
</tr>
<tr>
<td>Nevirapine</td>
<td>16.3 ± 2.60 (1)</td>
<td>2,211 ± 780 (136)</td>
<td>544 ± 90.0 (33)</td>
</tr>
</tbody>
</table>

* NL4.3 was used as the wild-type virus for the drug resistance assays. The amino acid substitutions (or deletion) in the RT of HIV_R1310D are V35I, S48G, T69G, K70R, L74I, Q102K, K103N, H135M, C162S, G196E, T215F, K219Q, R277K, R284K, V291I, and E297A. HIV_R1310D+D67 is HIV_R1310D with an aspartic acid (D) at RT codon 67.

* Data are means ± standard errors. The values were derived from at least three independent assays. Values shown in parentheses represent fold differences in IC_{50} compared to that of wild-type virus.

### Table 3. Drug susceptibilities of HIV constructs

<table>
<thead>
<tr>
<th>Construct</th>
<th>AZT</th>
<th>Nevirapine</th>
<th>ddC</th>
<th>ddI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>16.2 ± 2.60 (1)</td>
<td>25.2 ± 14.8 (1.0)</td>
<td>245 ± 157 (1.0)</td>
<td>925 ± 240 (3.8)</td>
</tr>
<tr>
<td>Δ</td>
<td>17.2 ± 8.35 (1.2)</td>
<td>445 ± 113 (18)</td>
<td>2,560 ± 1,160 (10)</td>
<td>ND</td>
</tr>
<tr>
<td>G</td>
<td>14.4 ± 3.84 (0.9)</td>
<td>270 ± 42.5 (11)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Δ+G</td>
<td>16.4 ± 3.45 (1.0)</td>
<td>82.8 ± 33.3 (3.3)</td>
<td>216 ± 76.7 (0.9)</td>
<td>ND</td>
</tr>
<tr>
<td>GN</td>
<td>600 ± 140 (37)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Δ+GN</td>
<td>1,770 ± 880 (109)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>RFO</td>
<td>142 ± 42.4 (8.3)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Δ+RFO</td>
<td>162 ± 23.0 (9.5)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>RFOG</td>
<td>146 ± 38.9 (8.5)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Δ+RFOG</td>
<td>7,610 ± 4,530 (445)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>RFOGNI</td>
<td>445 ± 78.3 (27)</td>
<td>327 ± 112 (13)</td>
<td>2,020 ± 662 (8.2)</td>
<td>397 ± 144 (1.6)</td>
</tr>
<tr>
<td>Δ+RFOGNI</td>
<td>1,768 ± 474 (108)</td>
<td>110 ± 24.8 (4.4)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Wild type, NL4.3; Δ, a deletion at RT codon 67; G, T69G; Δ+G, Δ of T69G; GN, T69G K103N; Δ+GN, Δ of T69G K103N; RFO, K70R T215F K219Q; Δ+RFO, Δ of K70R T215F K219Q; RFOG, K70R T215F K219Q T69G; Δ+RFOG, Δ of K70R T215F K219Q T69G; RFOGNI, K70R T215F K219Q T69G K103N L74I; Δ+RFOGNI, Δ of K70R T215F K219Q T69G K103N L74I.

* Data are means ± standard errors. Each construct was independently studied at least three times. Values in parentheses represent the fold differences in IC_{50} compared to that of wild-type virus.

* ND, not done.
HIV<sub>RT39180</sub> showed extremely high-level resistance to AZT (1,450-fold) in the absence of resistance to IND. Therefore, this decreased sensitivity to AZT of HIV<sub>RT39180</sub> was caused by drug resistance and was not due to a nonspecific characteristic of this chimeric virus.

Prior studies have identified high levels of AZT resistance as being in the range of 100- to 350-fold differences in IC<sub>50</sub> compared to that of the wild type and in association with amino acid substitutions M41L, D67N, K70R, L210W, T215F, and K219Q (19, 21, 24, 28, 30, 44). To further define the effect of the deletion at codon 67 on AZT susceptibility in this chimeric clone, the wild-type codon GAC (aspartic acid [D]) was inserted at RT codon 67. The resulting virus, HIV<sub>RT39180</sub> + D67, showed reduced IC<sub>50</sub> of AZT and nevirapine (26, 35) and increased IC<sub>50</sub> of ddI and ddC (Sigma) (Table 2). These results suggest that the presence of the deletion at codon 67 was an important component in conferring high-level resistance to AZT.

Drug susceptibility of HIV constructs. To assess the impact of the deletion alone on susceptibility to RT inhibitors and the potential interaction of the deletion with other amino acid substitutions, including known drug resistance mutations, a series of recombinant viruses were constructed by site-directed mutagenesis (Table 3). Constructs containing only the deletion at codon 67 (Δ) or only T69G (G) showed reduced susceptibility to ddC and ddI, but not to AZT or nevirapine. A construct containing the deletion and T69G (Δ+G) showed diminished sensitivity to only ddC (Table 3). However, the presence of Δ+G in the context of known AZT resistance mutations K70R, T215F, and K219Q (Δ+RFQG) led to a 445-fold increase in AZT resistance. Although addition of either of the single amino acid mutations K103N or L74I in the context of Δ + RFQG showed little effect on AZT susceptibility (data not shown), the presence of both mutations in the context of the Δ + RFQG motif increased the resistance to AZT to 1,813-fold (Table 3).

A mutant containing K103N alone showed 150-fold resistance to nevirapine (data not shown). Addition of the Δ67 deletion to the K103N mutant did not result in any significant change in the sensitivity to nevirapine (data not shown). The addition of the T69G change to the K103N mutant (GN) decreased the sensitivity to nevirapine from 150-fold to 37-fold (Table 3), suggesting that the T69G mutation can influence sensitivity to NNRTIs. The presence of the Δ67 deletion in the context of the T69G and K103N mutations led to an approximately threefold increase in sensitivity to nevirapine (Table 3). While the Δ67 deletion increased sensitivity to nevirapine, given the fact that different NNRTIs possess distinct binding specificities (4, 12, 23), the impact of this deletion on delavirdine or efavirenz sensitivity remains unclear. In order to elucidate the mechanism for this marked increase in AZT resistance, an additional series of HIV constructs were created (Fig. 2). A construct containing the deletion T69G and L74I (Δ+GI) showed only a fivefold increase in the IC<sub>50</sub> of AZT (93.7 ± 11.1 nM). A construct containing the deletion T69G, L74I, and K103N (Δ+GNI) showed only an 18-fold increase in IC<sub>50</sub> (305 ± 43.9 nM). Thus, the high-level resistance to AZT observed in Δ+RFQGNI was due to a synergistic interaction between the Δ+GNI and K70R T215F K219Q mutations (RFQ). It has been reported that several of the mutations associated with NNRTIs, such as Y181C and L100I, suppress AZT resistance (5, 29). Thus far, however, none of the mutations associated with NNRTI have been reported to enhance AZT resistance. K103N is one of the mutations that is commonly seen in the context of NNRTI resistance (44). It has been reported that this mutation has no impact on preexisting AZT resistance (5). In the present study, the presence of K103N and L74I conferred high-level resistance to AZT in the presence of the Δ67 deletion, and thus, these mutations can be categorized as mutations associated with AZT resistance under certain circumstances.

Further studies are needed to develop new approaches for impeding the development of these highly-drug-resistant forms of HIV-1 and to identify a salvage regimen or regimens.

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