Phenotypic and Genotypic Analysis of Biologically Cloned Human Immunodeficiency Virus Type 1 Isolates from Patients Treated with Zidovudine and Lamivudine

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Mutations at reverse transcriptase codons 44, 118, 207, and 208 were significantly correlated with reduced zidovudine susceptibility in biologically cloned human immunodeficiency virus type 1 (HIV-1) isolates. Sequences from the Stanford HIV RT and Protease Sequence Database showed that these mutations were more common in HIV-1 isolates from patients treated with zidovudine and lamivudine than in patients not treated with these drugs.

The combination of zidovudine (ZDV) and lamivudine (3TC) remains effective at inhibiting the replication of human immunodeficiency virus type 1 (HIV-1) despite the rapid emergence of 3TC resistance. This effectiveness is based in part on the interaction between the 3TC resistance mutation at reverse transcriptase (RT) codon 184 and ZDV resistance mutations. Introduction of the M184V substitution into strains carrying ZDV resistance mutations results in reversal of ZDV resistance (18), whereas coadministration of ZDV and 3TC results in delayed emergence of ZDV resistance mutations (9, 11). However, dually resistant isolates have been recovered from patients failing ZDV-3TC therapy (3, 14).

Mutations at both the 5′ and 3′ ends of the RT gene modulate expression of dual resistance [15; S. D. Kemp and S. Bloor, Antivir. Ther. 2(Suppl. 5):21-22, abstr. 11, 1997]. In addition, a G333E substitution promotes dual resistance in some, but not all, strains that harbor the classical ZDV and 3TC resistance mutations (8). To investigate further the genetic basis of dual resistance to ZDV and 3TC, we conducted a comprehensive clonal analysis of the phenotypic and genotypic characteristics of HIV-1 isolates from patients on prolonged treatment with these drugs.

Isolation and characterization of biological clones. Primary HIV-1 isolates resistant to ZDV and 3TC were cultured on peripheral blood mononuclear cells at limiting dilutions to generate independent biological clones (3). Susceptibilities of the clonal isolates to ZDV and 3TC were determined as described previously (6, 7). Resistance to ZDV was defined as intermediate (ZDVi; IC50 of 0.1 μM (10). Thirty biological clones were generated from 10 ZDVr 3TCs and 4 ZDVr 3TCs primary HIV-1 isolates, which were originally obtained from nine patients (3). The following phenotypes were observed: ZDVr 3TCs (eight clones), ZDVr 3TCi (five clones), ZDV-sensitive (ZDVs) 3TCr (nine clones), ZDVr 3TC-sensitive (3TCs) (three clones), ZDVr 3TCc (three clones), and ZDVr 3TCs (two clones) (Table 1).

Although primary isolates were selected for biological cloning on the basis of dual resistance to ZDV and 3TC, a minority of clones showed the ZDV- and 3TC-resistant phenotype. This observation most likely is explained by the outgrowth of sensitive viruses that constituted a minority of the viral quasispecies and suggests that ZDV- and 3TC-resistant isolates have a growth disadvantage compared to singly resistant or wild-type viruses. The possibility that some of the observed heterogeneity was due to the appearance or reversion of mutations during virus culture cannot be excluded.

Genotypic analysis of biological clones. The entire RT-coding sequence was amplified by a nested PCR from HIV-infected peripheral blood mononuclear cell DNA obtained at the end of culture and cloned into a PCR2.1 vector (Invitrogen, Carlsbad, Calif.). For plasma samples, viral RNA was extracted using the QIAamp viral RNA kit (QIAGEN) and reverse transcribed using the avian myeloblastosis virus reverse transcriptase system (Gibco/BRL, Gaithersburg, Md.) with primer TSTRSA1 (5′-CTATTTGGCTATCTTGAGGAC-3′, nucleotide [nt] 3965 corresponding to the HIV-1 Hxb2R sequence [http://hiv-web.lanl.gov]). First- and second-round PCR included the following steps: (i) incubation for 3 min at 95°C; (ii) 30 cycles, with 1 cycle consisting of 1 min at 94°C, 30 s at 58°C, and 2 min at 72°C; and (iii) 10-min extension phase at 72°C. For the first-round PCR, primer pair TSTRS1 (5′-ATGATAGGGG GAATTGGAGG-3′, nt 1934) and TCSRTA1 was used. The product of the first round was then amplified with primers TSTRS2 (5′-GCAAAAGCTTAGTAGGACCTACACC-3′, nt 1934) and TCSRTA1 was used. The product of the first round was then amplified with primers TSTRS2 (5′-GCAAAAGCTTAGTAGGACCTACACC TGTGC-3′, nt position 2024) and TCSRTA2 (5′-CGTTTGT CGACCTTGGGCCTATCTATCC-3′, nt 3803). The full-length RT-coding sequence of three independent molecular
clones from each biological clone was determined using an ABI 373A or ABI Prism 377 DNA automated sequencer (Perkin-Elmer, Foster City, Calif.). Sequences were manually aligned using BioEdit (T. A. Hall, Department of Microbiology, North Carolina State University, Raleigh), and a consensus sequence for each biological clone was generated.

Various combinations of ZDV- and 3TC-associated resistance mutations were observed (Table 1). Many clones carried additional RT substitutions including 44D, 118I, 196E, 207D or 207E (207D/E), 208Y, and 211K. None of the clones had the G33E mutation. Six clones derived from patient R001 carried the 151M multinucleotide resistance mutation and were excluded from subsequent analyses. RT sequences of the biological clones generally showed close concordance with virus
sequences from plasma samples obtained at the same time point.

**Association of RT mutations with ZDV susceptibility.** To determine the association of specific RT mutations on ZDV susceptibility, the geometric mean IC_{50} of biological clones with and without those mutations were compared. Because the independence of multiple clones obtained from an individual patient could not be assumed, a mixed-effects model was applied using SAS (SAS Institute Inc., Cary, N.C.). The antilog of the model-based mean and 95% confidence intervals were used to calculate the reported geometric means and 95% confidence intervals (95% CI).

Table 2 shows the geometric mean IC_{50} and 95% CI for ZDV and 3TC resistance mutations. The geometric mean IC_{50} for ZDV resistance mutations was found to be higher than the wild-type clones (Table 2). The 44D mutation and the 211K polymorphism were associated with twofold-higher geometric mean IC_{50} for ZDV, but these findings were marginally significant after the data were adjusted for multiple comparisons.

Although a large number of biological clones were analyzed in this study, they were derived from primary isolates obtained from only nine patients. Therefore, conclusions regarding possible associations of specific RT mutations with phenotype must be considered preliminary. To extend our findings, we sought to determine the frequency of these mutations in HIV-1 sequences from patients treated with ZDV and 3TC and patients not treated with nucleoside RT inhibitors. RT sequences from 218 unique ZDV- and 3TC-treated patients and 233 untreated patients were identified in the Stanford HIV RT and Protease Sequence Database (http://hivdb.stanford.edu/hiv/index.asp), and the occurrence of specific mutations was tabulated. Mutation frequencies in viral sequences from ZDV- and 3TC-treated and untreated patients were compared using mutation odds ratios that were programmed using Splus (MathSoft, Inc., Seattle, Wash.). The 44D, 118I, 207D/E, and 208Y mutations were significantly more likely to be present in RT sequences from patients treated with ZDV and 3TC than in sequences from patients not treated with these drugs (Table 3).

The results of several studies point to the potential significance of mutations at codons 44 and 118 in patients treated with nucleoside RT inhibitors (2, 5, 16, 17). Our finding that the 44D/E and 118I mutations were more common in viruses from ZDV- and 3TC-treated patients is consistent with these results. An earlier study analyzed the genotype and phenotype of molecularly cloned RT sequences derived from isolates from four patients receiving ZDV and 3TC combination therapy (15). In agreement with the data presented here, multiple ZDV resistance mutations were found to be required for a dual-resistance phenotype. Mutations at codons 44, 118, 196, 207, 208, and 211 were present in clones from two of four patients in that study, but the potential significance of those mutations was not discussed.

Biological clones that carried the 207D/E mutation had a geometric mean IC_{50} for ZDV that was 22.5-fold greater than clones that had the wild-type codon at this position (P < 0.001). The potential importance of mutations at this codon for viral adaptation to replication in the presence of ZDV is supported by our analysis of data from the Stanford HIV RT and Protease Sequence Database, which showed that the 207D/E mutations were significantly more likely to be present in HIV-1 sequences from ZDV- and 3TC-treated patients than in sequences from untreated control patients (Table 3). Similarly, the H208Y mutation was associated with a 17-fold-higher geometric mean IC_{50} for ZDV. Of note, the 208Y mutation was first reported in association with foscarnet resistance (12). Serial in vitro passage of HIV-1 in the presence of foscarnet led to emergence of the G161L and 208Y mutations, which together conferred approximately eight- to ninefold-higher resistance to foscarnet. When present together with 161L, the 208Y mutation enhanced susceptibility to ZDV, whereas the results of our study suggest the opposite effect when this mutation was present together with ZDV resistance mutations at codons 41, 67, 70, 210, 215, and 219.

Studies from several laboratories have shown that ZDV resistance mutations enhance the removal of the terminal ZDV monophosphate from the growing primer chain by pyrophosphate or ATP, thereby relieving the block to reverse transcription (1, 13). By contrast, the 184V mutation reduces the rate of pyrophosphorolysis and decreases primer unblocking (4). It will be important to determine the mechanism by which mutations described in this report alter ZDV susceptibility and to determine whether mutations that confer dual resistance to ZDV and 3TC are critical for the emergence of these resistance mutations.
ZDV and 3TC restore primer unblocking activity despite the presence of 184V or alter the kinetics of ZDV-TP binding to the enzyme-primer/template complex.

In conclusion, results of this study suggest that substitutions at RT codons 44, 118, 207, and 208 modulate the expression of ZDV resistance in HIV-1 isolates that carry ZDV resistance mutations at codons 41, 67, 70, 210, 215, and/or 219 with or without the 3TC resistance mutation at codon 184. These findings may have practical importance in helping to refine the interpretation of genotypic resistance data regarding ZDV and 3TC in samples from patients who have received treatment with both drugs.

Nucleotide sequence accession numbers. Consensus RT sequences from biologically cloned HIV-1 isolates were deposited in GenBank data bank under accession numbers AY013828 to AY013868.

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REFERENCES


