Drug-Selected Resistance Mutations and Non-B Subtypes in Antiretroviral-Naive Adults with Established Human Immunodeficiency Virus Infection

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The prevalence of human immunodeficiency virus (HIV) type 1 antiretroviral resistance is expected to be higher in recently infected antiretroviral-naive individuals than in those who have been infected longer. Antiretroviral-naive HIV-1–infected adults who presented to an outpatient clinic in an urban hospital in Boston for initial evaluation in 1999 were screened for drug-selected resistance mutations and phylogenetic subtype. Drug-selected mutations were identified in 16 (18%) of 88 subjects. Twelve (14%) included mutations associated with nucleoside reverse-transcriptase inhibitors, 4 (5%) included mutations associated with non-nucleoside reverse-transcriptase inhibitors, and 3 (3%) included mutations associated with protease inhibitors. Two (2%) had resistance mutations associated with multiple classes of drugs. Nine (10%) subjects had infection with non-B subtype HIV-1 and did not have drug-selected mutations. Serological results indicated infection for ≥6 months. Drug-selected mutations or non-B subtypes were detected in a substantial portion of antiretroviral-naive adults who had been infected for at least 6 months.

Drug-selected resistance mutations in the human immunodeficiency virus (HIV) type 1 polymerase gene (pol), which includes nucleotide sequences for viral protease and reverse transcriptase (RT), cause decreased phenotypic drug susceptibility in vitro and are associated with clinical treatment failure [1, 2]. The transmission of drug-selected resistance mutations has been documented for all currently approved classes of antiretroviral agents. The prevalence of genotypic resistance in newly infected patients (i.e., those infected within ~6 months of genotypic testing) may be as high as 26% [3–5]. However, data are limited for antiretroviral-naive patients who have been infected for longer durations. Resistance testing is recommended after treatment failure and should be considered before the initiation of therapy in patients with acute HIV-1 infection [2]. Resistance testing currently is not routinely recommended for pretreatment screening in antiretroviral-naive patients who have been infected for longer durations. Resistance testing is recommended after treatment failure and should be considered before the initiation of therapy in patients with acute HIV-1 infection [2]. Resistance testing currently is not routinely recommended for pretreatment screening in antiretroviral-naive patients who have been infected for longer durations, because it is assumed that resistance mutations will be less commonly detected than in cases of acute infection [2].

Aside from resistance issues, the predominant form of HIV-1 circulating in the United States has been group M, subtype B. Although an increasing prevalence of

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non-B subtypes of HIV-1 in Europe has been documented by some studies, the few available reports have suggested that non-B subtypes were relatively rare in the United States through 1999 [6, 7]. Our objectives were to determine the prevalence of drug-selected mutations and non-B subtypes in chronically infected HIV-1–positive adults in Boston who had no antiretroviral drug experience and presented for initial evaluation in 1999.

SUBJECTS AND METHODS

HIV-1–infected patients newly presenting for HIV care in 1999 at an urban Boston hospital (Boston Medical Center) were prospectively screened, to identify individuals who were antiretroviral naive. Inclusion criteria for the study were HIV-1 antibody positivity and no history of prior antiretroviral therapy. Demographic data and blood samples were collected at the initial visit. Blood specimens from the initial visit (before starting any antiretroviral medications) were used for all laboratory studies. A quantiplex HIV-1 RNA 3.0 assay (bDNA; Bayer) was used to quantify plasma HIV-1 RNA levels. Plasma HIV-1 genotypic analysis was done retrospectively on frozen plasma samples with HIV-1 RNA levels ≥1000 copies/mL. The institutional review board at Boston Medical Center approved the study, and all participants gave informed consent.

Coded specimens were also evaluated by the serological testing algorithm for recent HIV seroconversion (STARHS), which uses a modified EIA to determine whether HIV-1 infection was recent (i.e., within ~6 months of testing) or more long term (≥6 months’ duration) [8]. The Organon Teknika Vironostika HIV-1 EIA sensitivity was decreased by using a sample dilution of 1:20,000 and an incubation time of 30 min, to create a less sensitive EIA (Vironostika-LS). Positivity in both the standard and the modified EIA indicated an estimated time to seroconversion of ≥170 days (95% confidence interval [CI], 162–183 days) for HIV-1 subtype B (Centers for Disease Control and Prevention, correspondence and protocol).

Total RNA was extracted from plasma using the QIAamp Viral RNA Mini Kit (Qiagen). RNA was used for reverse-transcription polymerase chain reaction (PCR) using the HIV-1 TruGene kit (Visible Genetics). The PCR product was sequenced and analyzed using an automated cycle sequencer (Visible Genetics). Double-stranded confirmation of the nucleotide sequence was obtained for all of protease (codons 1–99) and RT codons 38–247. All specimens yielded adequate sequence data. Nucleotide sequences have been submitted to GenBank (accession nos. AY049528–AY049703).

The nucleotide sequences we obtained were compared with each other, with sequences from other specimens analyzed during the same period, and with laboratory strains of HIV-1, to exclude PCR cross-contamination or specimen misidentification. Pairs of plasma specimens with HIV-1 pol nucleotide sequences that were ≥98% identical or ≥99% similar to each other were identified. These specimens underwent repeated RNA extraction, reverse transcription–PCR, and nucleotide sequencing, to rule out cross-contamination. These specimens were also tested with an antibody-profile Western blot assay (Viral and Rickettsial Disease Laboratory, California Department of Health Services, Berkeley, CA) that distinguishes specimens from different persons, to rule out specimen misidentification [9].

Antiretroviral drug resistance mutations reported here are based on published results [1, 2]. These mutations are all associated with decreased phenotypic susceptibility to an antiretroviral drug when present either individually or on a specific genetic background of other resistance mutations. For our analysis, we included only resistance mutations that are specifically selected by antiretroviral drug therapy (here called “drug-selected resistance mutations”). Mutations that are known to occur naturally in viruses infecting drug-naive patients (i.e., naturally occurring polymorphisms, such as most secondary protease inhibitor [PI] resistance mutations) were not included in our analysis even if they also contribute to resistance when they are present with other resistance-associated mutations. Nucleoside and nucleotide RT inhibitor (NRTI) resistance mutations included RT M41L, E44D, A62V, K65R, D67N, T215Y/F, K219Q/E, and any insertions or deletions in the vicinity of RT codon 69. Nonnucleoside RT inhibitor (NNRTI) resistance mutations included RT A98G, L100I, K101E, K103N, V106A, V108I, Y181C/I, Y188C/L/I, G190A/S, P225H, and P236L. PI resistance mutations included protease D30N, M46I/L, G48V, I50V, V82A/F/T, I84V, and L90M and insertion mutations in the vicinity of codon 36.

Sequences were screened for subtype and recombinant forms (HIV-1 Genotyping Tool; NCBI, available at http://www.ncbi.nlm.nih.gov/refseqhivGenotypes/subtype/subtype.html) and predicted genotypes that were later confirmed by phylogenetic analysis (Wisconsin Package version 10.2; Genetics Computer Group). Sequences were aligned and gap-stripped to yield a consensus length of 920 bp, and phylogenetic trees were inferred by using the neighbor-joining method and the Kimura 2-parameter model. Bootstrapping was done using 100 replicates.

RESULTS

During 1999, 115 newly presenting adults met the inclusion criteria (HIV-1 antibody positive with no history of prior antiretroviral therapy), and all 115 consented to enroll in the study. Of these, 88 had frozen stored plasma samples available for genotyping. We were able to amplify and perform genotyping successfully on samples from all 88 subjects.
The cohort had a median age of 37 years (range, 18–62 years) and included 29 (33%) women. The cohort was racially and ethnically diverse, with 65% African Americans or Africans, 23% whites, and 11% Latinos. Thirty-one percent had previously lived outside the United States (most commonly in the Caribbean Basin or Africa). Most (51%) had heterosexual activity as their only risk factor for HIV-1 acquisition. Other risk groups included men who had sex with men (MSM; 20%) and injection drug users (IDUs; 31%); 2 individuals were in both the MSM and IDU risk groups. Thirty (34%) had anti-hepatitis C virus antibodies. As would be expected in adults presenting with chronic infection, there was a wide range in baseline CD4 T cell counts (median, 297 cells/mm³; range, 5–997 cells/mm³) and plasma HIV-1 RNA levels (median, 51,610 copies/mL; range, 1107 to >500,000 copies/mL). STARHS analyses using the Vironostika-LS assay were done on 78 specimens of plasma that remained after the initial preplanned genotypic analyses. Only 1 of these was scored as an incident sample (e.g., <6 months since virus acquisition).

Sixteen subjects (18%; 95% CI, 10%–26%) were infected with strains of HIV-1 that carried drug-selected resistance mutations (table 1). All viruses with these mutations belonged to group M subtype B (results not shown). All 16 subjects harboring virus with drug-selected mutations were scored by STARHS as having more long-term HIV-1 infections (e.g., >6 months since virus acquisition), not incident or recent infection. Furthermore, 13 of 16 individuals with drug-selected mutations had CD4 T cell counts ≤500 cells/mm³, a further indication of long-term infection. There were no trends or significant differences in the prevalence of viruses with drug-selected mutations according to HIV-1 risk factors or other demographic or laboratory variables (results not shown).

Viruses in 12 subjects (14%) included mutations associated with NRTI resistance. Two had mutations at RT codons 41 and 210, which were associated with resistance to zidovudine, stavudine, abacavir, and tenofovir [2, 10]. One had a mutation at codon 74 that was associated with resistance to didanosine, zalcitabine, and abacavir. Five had mutations at RT codon 69 that were associated with resistance to multiple NRTIs [11]. Viruses from 4 individuals had mutations at codons 44 and 118 that were associated with resistance to lamivudine [2]. One subject had virus with a mutation at codon 115 that was associated with abacavir resistance [2].

Two of these subjects also had changes at RT codon 215 (T215C, T215D, and T215E) that were different from the resistance-associated RT T215Y or T215F. These changes are seen in viruses from individuals who are initially infected with virus carrying the resistance mutations but do not undergo therapy [12]. Changes in RT codon 215 were seen only in viruses with other NRTI resistance mutations.

Table 1. Antiretroviral-naive subjects infected with human immunodeficiency virus (HIV) type 1 with drug resistance–associated mutations.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Plasma HIV-1 RNA, copies/mL</th>
<th>Risk factor</th>
<th>Country of origin</th>
<th>pol mutation conferring resistance to</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PIs</td>
</tr>
<tr>
<td>G012</td>
<td>&gt;500,000</td>
<td>IDU</td>
<td>United States</td>
<td>M41L, L210W, T215E/D</td>
</tr>
<tr>
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<td>50,135</td>
<td>Heterosexual</td>
<td>United States</td>
<td>T69N</td>
</tr>
<tr>
<td>G018</td>
<td>47,947</td>
<td>MSM</td>
<td>United States</td>
<td>T69N/T</td>
</tr>
<tr>
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<td>United States</td>
<td>M36DI</td>
</tr>
<tr>
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<td>55,498</td>
<td>Heterosexual</td>
<td>El Salvador</td>
<td>L90M</td>
</tr>
<tr>
<td>G035</td>
<td>1609</td>
<td>Heterosexual</td>
<td>United States</td>
<td>L90M/L</td>
</tr>
<tr>
<td>G052</td>
<td>1556</td>
<td>Heterosexual</td>
<td>United States</td>
<td>Y115Y/F</td>
</tr>
<tr>
<td>G053</td>
<td>168,642</td>
<td>Heterosexual</td>
<td>United States</td>
<td>V118I/V</td>
</tr>
<tr>
<td>G060</td>
<td>&gt; 500,000</td>
<td>MSM</td>
<td>United States</td>
<td>K103N</td>
</tr>
<tr>
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<td>K101K/E</td>
</tr>
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<td>11,064</td>
<td>MSM</td>
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<td>T69S</td>
</tr>
<tr>
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<td>3105</td>
<td>Heterosexual</td>
<td>Haiti</td>
<td>V118I</td>
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<td>51,123</td>
<td>IDU</td>
<td>United States</td>
<td>T69N/T</td>
</tr>
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<td>IDU</td>
<td>United States</td>
<td>E44D</td>
</tr>
<tr>
<td>G087</td>
<td>92,596</td>
<td>IDU</td>
<td>United States</td>
<td>M41L, L74V, T215C, Y181C</td>
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<tr>
<td>G090</td>
<td>121,275</td>
<td>IDU</td>
<td>United States</td>
<td>V118I</td>
</tr>
</tbody>
</table>

NOTE. IDU, injection drug use; MSM, men who have sex with men; NNRTIs, nonnucleoside reverse-transcriptase inhibitors; NRTIs, nucleoside reverse-transcriptase inhibitors; PIs, protease inhibitors; pol, polymerase gene.

* Mutations shown in italics are at resistance-associated codons and may represent partial revertants in the absence of drug-selection pressure.
Viruses in 4 subjects (5%) included mutations associated with NNRTI resistance [2]. One (G060) had genotypic resistance to all 3 approved drugs in this class. Two had mutations associated with resistance to 2 NNRTIs (G034, with resistance to nevirapine and efavirenz, and G087, with resistance to nevirapine and delavirdine), and 1 (G061) had a mutation associated with nevirapine resistance.

Viruses in 3 subjects (3%) included mutations associated with PI resistance, including 2 with a primary resistance mutation for saquinavir and nelfinavir (protease L90M) that may also contribute to resistance to other PIs [2]. One had a newly described insertion mutation in the vicinity of protease codon 37 that develops after the initiation of PI therapy and may be associated with virological failure to PIs [13].

Viruses in 2 subjects (2%) had resistance mutations associated with multiple classes. One had mutations causing resistance to both NRTIs and NNRTIs. Another had mutations conferring resistance to all 3 currently approved classes of antiretroviral agents.

Phylogenetic analyses were done to evaluate the prevalence of non-B subtype virus and whether drug-selected resistance mutations were found in non-B subtypes. Viruses in 9 (10%) of 88 subjects (95% CI, 4%–16%) had pol sequences that were entirely non-B in subtype (figure 1). None of these had any drug-selected resistance mutations. All 9 subjects had previously lived outside the United States. There were 4 subtype A viruses (in subjects from Uganda) and 2 subtype C viruses (in subjects from Ethiopia). Viruses from 3 other subjects had pol regions that grouped in recognized circulating recombinant forms (CRFs). Viruses in 2 subjects (1 from Cameroon and 1 from Nigeria) grouped with CRF02 (A/G recombinant). Another virus in a subject from Vietnam grouped with CRF01 (A/E recombinant).

Three pairs of plasma specimens had HIV-1 pol nucleotide sequences that were ≥98% identical or ≥99% similar to each other: G005 and G009 (98.8% identical and 99.8% similar), G043 and G073 (97.3% identical and 99.6% similar), and G079 and G080 (97.3% identical and 99.2% similar). Repeated RNA extraction, reverse transcription–PCR, and nucleotide sequencing of these specimens ruled out cross-contamination within the testing laboratory (results not shown). These specimens were also tested with an antibody-profile Western blot assay that was used to distinguish among different persons [9]. Each of these 6 specimens had a unique antibody profile that excluded the possibility of specimen misidentification. An epidemiological linkage within each of the 3 pairs of subjects, however, cannot be ruled out. In contrast, specimens with resistance mutations were phylogenetically different enough from each other (≥95.5% identical and ≥97.8% similar for each pairwise comparison) as to suggest that an epidemiological linkage among them was not likely.

**DISCUSSION**

Drug-selected resistance mutations in HIV-1 pol were detected in 18% of antiretroviral-naive adults who did not have recent HIV-1 acquisition and who entered care in a single clinic in Boston during 1999. This is similar to the prevalence of genotypic resistance (5%–26%) noted in studies that included only recently infected antiretroviral-naive individuals in the United States [3–5]. A preliminary report on 603 recently diagnosed antiretroviral-naive persons in 10 US cities in 1997–2000 showed that 7.5% had a virus strain with drug-selected resistance mutations [14]. No difference between more chronically infected and recently infected subgroups was seen [14].

Our findings suggest that strains of HIV-1 with drug-selected resistance mutations are actively circulating and should no longer be considered rare, even in unselected patients with an unknown duration of infection. Resistance testing is currently recommended for drug-experienced patients for whom therapy is not successful and is not routinely recommended for treatment-naive patients with established infection, to detect the prior transmission of drug-resistant virus [2]. Pretreatment testing is suggested more strongly for patients presenting with acute HIV-1 infection than for those with unknown (and likely longer) durations of infection [2]. Some reports have suggested that the virological and immunological responses to therapy are poorer in treatment-naive patients who start therapy for acute HIV-1 infection with a regimen to which their virus has preexisting resistance [5]. The results of a recent analysis have shown that genotypic resistance testing (done similarly to our methods) is likely to be cost effective if the prevalence of resistance mutations in treatment-naive patients with either acute or chronic infection is at least 4%, which is well below the prevalence found in our patient population [15]. The screening antiretroviral-naive patients with established infections of unknown duration for genotypic resistance prior to starting therapy merits further study, to determine whether it may be appropriate for clinical management in areas with at least that level of prevalence of resistance.

A substantial minority of patients in our study had infection with non-B subtypes of HIV-1, in contrast to the results of previous studies, which have suggested that non-B subtypes are rare in the United States [6, 7]. The prevalence seen in our cohort should prompt clinicians to consider the possibility of non-B subtype infection, particularly among populations that include persons from Africa or Asia.

In summary, we found that, among antiretroviral-naive adults entering care with established HIV-1 infection in a clinic in Boston in 1999, 18% had infection with subtype B virus with important drug-selected resistance mutations, and 10% were infected with non-B subtypes of HIV-1. Further studies are needed in other locales to determine the prevalence of HIV-1 drug-selected mutations in antiretroviral-naive persons with
Figure 1. Phylogenetic analysis of a subgenomic pol region from human immunodeficiency virus (HIV) type 1 non-B subtype specimens. Only specimens with non-B subtype pol sequences (in bold italic type) were included in the analysis. All other sequences represent reference sequences of groups N and O and different subtypes or circulating recombinant forms (CRFs) of HIV-1 group M and were obtained from the Los Alamos database (available at http://hiv-web.lanl.gov/). Tree topology was inferred from nucleotide sequences by the neighbor-joining method using the Kimura 2-parameter model. Nos. at a branch indicate the percentage of bootstrap values (of 100 replicates) in which the cluster to the right is supported. Only values ≥70% at pertinent nodes are shown. The scale bar shows the estimated ratio of nucleotide substitutions for a given horizontal branch length; vertical distances are for clarity only. G017 and G030 grouped in subtype C; G008 and G039 grouped in CRF02-AG; G051 grouped in CRF01-AE; and G014, G040, G064, and G100 grouped in subtype A.
both established and recent infection and the optimal diagnostic and treatment strategies for these patients.

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References


Addendum. After submission of the manuscript, updated guidelines that were based on the results of new studies, including ours, were published that suggested pretreatment screening for certain patients who have been infected for as long as ≥2 years before starting therapy [16].