HIV-1 Drug Resistance in Newly Infected Individuals

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Context There is concern that the widespread use of antiretroviral drugs to treat human immunodeficiency virus 1 (HIV-1) infection may result in the increased transmission of drug-resistant virus.

Objective To determine the prevalence of drug resistance–conferring mutations and phenotypic resistance to antiretroviral agents in a cohort of individuals newly infected with HIV-1.

Design Case series with genetic analyses of the HIV-1 plasma-derived pol gene using reverse transcriptase polymerase chain reaction followed by direct sequencing of polymerase chain reaction products. Phenotypic analysis was performed with a recombinant virus assay.

Setting and Patients Eighty individuals referred, on average, 1.7 months after infection with HIV-1 to the Aaron Diamond AIDS Research Center between July 1995 and April 1999. Subjects were from large urban areas (65 from New York, NY; 11 from Los Angeles, Calif); 60 (75%) were white, and 75 (93.8%) were homosexual men.

Main Outcome Measures Prevalence of known resistance-conferring genotypes and reduced susceptibility to individual antiviral agents by phenotype.

Results Thirteen individuals (16.3%) had genotypes associated with drug resistance to any antiretroviral agent. Virus with known resistance-conferring mutations to any nucleoside reverse transcriptase inhibitors was found in 10 individuals, to any nonnucleoside reverse transcriptase inhibitors in 6 subjects, and to any protease inhibitors in 2 cases. Multidrug-resistant virus was identified in 3 individuals (3.8%). Extensive polymorphism in the protease gene was identified. Interpretation of genotypes and phenotypes was concordant in 57 (85%) of the 67 cases in which both studies were performed.

Conclusion The prevalence of HIV-1 variants with known resistance-conferring genotypes to any antiretroviral agent in this cohort of 80 newly infected individuals is 16.3%. These data support expanded use of resistance testing in the setting of primary HIV-1 infection. Clinical trials should be initiated to establish whether therapy guided by resistance testing, compared with the use of empirical triple combination antiretroviral therapy, provides additional virological and immunological benefit when treating primary HIV-1 infection. Further efforts to expand the study of transmission of drug-resistant HIV-1 variants, particularly in cohorts with different epidemiological profiles, are indicated.

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able protease inhibitors (saquinavir, ritonavir, indinavir, and nelfinavir). Clearly, the transmission of resistant variants to uninfected individuals raises serious clinical and public health consequences. It has been hypothesized that the widespread use of antiretroviral drugs will result in the increased transmission of drug-resistant virus resulting in an increased prevalence of resistant variants in newly infected patients. To address this question, we determined the prevalence of mutations in the HIV-1 pol gene associated with resistance to antiretroviral agents in 80 individuals newly infected with HIV-1 between July 1995 and April 1999, a period during which the use of combinations of antiretroviral agents to treat HIV-1 infection became the standard of care.

**METHODS**

**Study Population**

Plasma samples from 80 individuals newly infected with HIV-1 were analyzed. Subjects were analyzed, on average, 1.7 months (range, 0.5-5.0 months) after primary infection. Criteria used to document newly acquired HIV infection included detectable HIV-1 RNA by either signal amplification (branched DNA, Chiron 2.0, Emeryville, Calif) or reverse transcriptase polymerase chain reaction (PCR) (Roche, Alameda, Calif) and either an absent or evolving humoral immune response to HIV-1 or a clinical history compatible with HIV-1 infection (within 90 days of presentation) and a documented negative HIV-1 serologic test result within 120 days of presentation to the Clinical Service of the Aaron Diamond AIDS Research Center (ADARC) of Rockefeller University, New York, NY. The patients reported here represent 80 of 82 consecutively identified newly HIV-1–infected individuals who were either physician- or self-referred to the ADARC for participation in early intervention clinical trials conducted between July 1995 and April 1999 (7 subjects in 1995, 28 in 1996, 23 in 1997, 15 in 1998, and 7 in 1999). Stored plasma samples were not available from 2 individuals infected and identified in 1993 and were therefore excluded from the analysis. Seventy-seven men and 3 women from large urban areas including New York (n = 65), Los Angeles, Calif (n = 11), San Francisco, Calif (n = 2), Houston, Tex (n = 1), and Toronto, Ontario (n = 1) are included. Most (75%) were white and 93.8% had become infected via sexual transmission (men having sex with men) (TABLE 1). Plasma HIV-1 RNA levels at presentation and at the time of resistance testing ranged from less than 500 to 6 × 10^6 HIV-1 RNA copies/mL (mean, 361 931 copies/mL). Informed consent was obtained from all subjects and human experimentation guidelines of the US Department of Health and Human Services and those of Rockefeller University Institutional Review Board were strictly followed.

**Genotypic Analysis of HIV-1 Reverse Transcriptase Gene**

Viral RNA was isolated from plasma with the QIAamp Viral Extraction Kit (Qiagen Inc, Chatsworth, Calif) according to the manufacturer’s instructions. The HIV-1 reverse transcriptase gene was reverse transcribed to complementary DNA (cDNA) at 48°C for 50 minutes by using HIV-1–specific downstream primer RT-3 (nucleotide position 3542-3559 of HIV-1 \text{pol}) and SuperScript II RNAse H reverse transcriptase (Gibco-BRL, Long Island, NY). A 1191–base pair (bp) reverse transcriptase gene fragment was amplified from cDNA by seminested PCR. First-round PCR was performed with upstream primer RT-1 (nucleotide position 2364-2383) and downstream primer RT-3 for 3 minutes at 94°C followed by 35 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1.2 minutes, with a final extension at 72°C for 10 minutes. Two microliters of the first-round product was used for second-round amplification with identical conditions with upstream primer RT-1 and downstream primer RT-2 (nucleotide position 3537-3554).

**TABLE 1. Baseline Characteristics of 80 Subjects With Primary HIV-1 Infection* **

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Male (%)</th>
<th>Female (%)</th>
<th>Race (%)</th>
<th>Sex (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>34.8 (6.5)</td>
<td>20-60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mode of infection, No. (%)</td>
<td>75 (93.8)</td>
<td>4 (5.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intravenous drug use</td>
<td>1 (1.3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial plasma HIV RNA level, copies/mL</td>
<td>1.70 (0.46)</td>
<td>0.2-5.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial CD4 cell count, ×10^9/L</td>
<td>361 931 (799 816)</td>
<td>&lt;500-6 × 10^6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* HIV-1 indicates human immunodeficiency virus 1.
vidual viral RNA molecules24-26 was determined as follows: cDNA was synthesized and 5-fold serially diluted and amplified by nested PCR under conditions as described herein. Four replicates of each dilution were amplified until an end point was reached in which no more than 2 of 4 replicates were positive. At this dilution, 20 replicates were performed to obtain 10 to 12 positive reactions for sequencing.

DNA Sequencing of the Protease and Reverse Transcriptase Genes

The DNA product of PCR amplification was column purified by using Qiaquick PCR kit (Qiagen Inc). Direct sequencing was performed with the ABI Prism 377 DNA Sequencer (Perkin Elmer, Norwalk, Conn). Cycle sequencing was carried out on the GeneAmp PCR System 9600 (Perkin Elmer) using AmpliTaq DNA polymerase and dye-labeled terminators (Perkin Elmer/Applied Biosystems, Foster City, Calif). Sequence primers R-3 (nucleotide position 2484-2504), R-4 (nucleotide position 2436-2453), R-6 (nucleotide position 3255-3279), and R-7 (nucleotide position 2811-2834) were used to analyze the amplified fragment of the reverse transcriptase gene (codons 1-318). The entire protease gene was sequenced with the inner PCR primers PT-2 and PT-3.

Sequence Analysis

Individual sequences from protease and reverse transcriptase gene obtained by direct DNA sequencing were manually edited and pairwise aligned to pNL4.3 as a the reference sequence using the EDITSEQ and MEGALIGN programs included in the Lasergene software package (DNASTAR Inc, Madison, Wis). The presence of resistance-conferring mutations was based on an extensive search of the HIV-1 resistance mutation database (Los Alamos National Laboratory, Los Alamos, NM). The phylogenetic analysis was performed using the distance matrix program DNADIST with the stochastic model of Kimura 2 and the NEIGHBOR program, both supplied in the PHYLPACK package (distributed by Joseph Felsenstein, PhD, University of Washington, Seattle).27

Phenotypic Analysis

In vitro drug susceptibility testing was performed with a recombinant virus assay (ViroLogic Inc, South San Francisco, Calif). Briefly, the protease and reverse transcriptase portion of the pol gene was amplified by reverse transcriptase (PCR) from plasma viral RNA samples. The amplified fragment was digested and ligated to a modified HIV-1 DNA vector that includes a luciferase reporter gene and is restricted to a single round of viral replication. Following transformation of the recombinant resistance test vectors into Escherichia coli, plasmid DNA representing the pool of virus present in the patient plasma was purified and used to transfect 293 cells. Viral particles were collected 48 hours after transfection and used to infect fresh cells, which were lysed after 48 hours and luciferase activity was measured. Protease inhibitors were added to transfected cells and reverse transcriptase inhibitors were added to infected cells. Drug susceptibility was measured by plotting the percentage of inhibition of luciferase activity against the log of the drug concentration to determine the concentration of drug that inhibited luciferase activity by 50%. Increased or decreased susceptibility to a particular drug is expressed as the fold-change between the patient sample concentration of drug that inhibited luciferase activity by 50% and that of a construct containing protease and reverse transcriptase from pNL4.3. Reproducibility studies have demonstrated that 95% of values from replicate assays vary by less than 2.5-fold. In this study, we selected up to a 3.0-fold change as the upper limit of susceptibility to any particular drug.

RESULTS

Genetic Analysis of HIV-1 Reverse Transcriptase and Protease Genes

Of the 80 plasma RNA samples studied, all could be amplified and analyzed by direct DNA sequencing. Virus from 10 subjects (12.5%) was found to harbor resistance-conferring mutations to NRTIs (Table 2). Six of these were found to harbor mutations associated with zidovudine resistance. Two subjects harbored virus with multiple resistance mutations in reverse transcriptase and protease, including zidovudine-related resistance mutations M41L (n = 2), D67N (n = 2), K70R (n = 1), L210W (n = 1), T215Y/F (n = 2), and K219Q (n = 1). Isolated zidovudine resistance mutations M41L (n = 2) and K70R (n = 2) could be identified in virus from 4 individuals. An isolated amino acid substitution, D67N, that is associated with resistance to zidovudine only in context with other resistance-conferring mutations (ie, M41L, K70R, and T215Y) (Brendan A. Larder, MA, PhD, oral communication, April 1999) was found in 5 isolates. The lamivudine-related resistance mutation M184V was detected in 4 individuals (5.0%), 3 by population consensus sequencing and 1 by limiting-dilution PCR.

Resistance-conferring mutations to nonnucleoside reverse transcriptase inhibitors (NNRTIs) were seen in 6 indi-

<table>
<thead>
<tr>
<th>Mutations</th>
<th>No. of Individuals (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary NRTIs, total</td>
<td>10 (12.5)</td>
</tr>
<tr>
<td>M41L</td>
<td>4 (5.0)</td>
</tr>
<tr>
<td>K70R</td>
<td>3 (3.8)</td>
</tr>
<tr>
<td>M184V</td>
<td>4 (5.0)</td>
</tr>
<tr>
<td>L210W</td>
<td>1 (1.3)</td>
</tr>
<tr>
<td>T215Y/F</td>
<td>1 (1.3)</td>
</tr>
<tr>
<td>K219Q</td>
<td>1 (1.3)</td>
</tr>
<tr>
<td>Secondary NRTI, total</td>
<td>7 (8.8)</td>
</tr>
<tr>
<td>Primary NNRTI, total</td>
<td>6 (7.5)</td>
</tr>
<tr>
<td>K103N</td>
<td>1 (1.3)</td>
</tr>
<tr>
<td>V179D/E</td>
<td>3 (3.8)</td>
</tr>
<tr>
<td>Y181H</td>
<td>1 (1.3)</td>
</tr>
<tr>
<td>Y188L</td>
<td>1 (1.3)</td>
</tr>
<tr>
<td>Primary protease inhibitors, total</td>
<td>2 (2.5)</td>
</tr>
<tr>
<td>V32A</td>
<td>1 (1.3)</td>
</tr>
<tr>
<td>L90M</td>
<td>2 (2.5)</td>
</tr>
<tr>
<td>Protease inhibitor polymorphisms, total</td>
<td>63 (78.8)</td>
</tr>
<tr>
<td>At residue 10</td>
<td>14 (17.5)</td>
</tr>
<tr>
<td>At residue 56</td>
<td>4 (5.0)</td>
</tr>
<tr>
<td>At residue 63</td>
<td>48 (60.0)</td>
</tr>
<tr>
<td>At residue 77</td>
<td>16 (20.0)</td>
</tr>
<tr>
<td>At residue 93</td>
<td>28 (35.0)</td>
</tr>
</tbody>
</table>

*HIV-1 indicates human immunodeficiency virus 1; NRTI, nucleoside reverse transcriptase inhibitor; and NNRTI, nonnucleoside reverse transcriptase inhibitor.
TRANSMISSION OF RESISTANT HIV-1

Figure 1. Distribution of Newly Acquired HIV-1 Infection

<table>
<thead>
<tr>
<th>Calendar Year of HIV-1 Infection</th>
<th>No. of Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>1995</td>
<td>10</td>
</tr>
<tr>
<td>1996</td>
<td>5</td>
</tr>
<tr>
<td>1997</td>
<td>20</td>
</tr>
<tr>
<td>1998</td>
<td>15</td>
</tr>
<tr>
<td>1999</td>
<td>5</td>
</tr>
</tbody>
</table>

HIV-1 indicates human immunodeficiency virus 1. Each column shows the number of newly infected individuals harboring HIV-1 with resistance-conferring mutations to nucleoside reverse transcriptase inhibitors alone (NRTIs) (pink), nonnucleoside reverse transcriptase inhibitors alone (NNRTIs) (blue), and multiple drug classes, including protease inhibitors (gray), and the number of those harboring HIV without resistance-conferring mutations (white).

Figure 2. Phylogenetic Tree Analysis of Genotypes of HIV-1 Strains

Data are based on the pol region of human immunodeficiency virus 1 (HIV-1) (nucleotide positions 2253-3217 of HIV-1pNL4.3) from 80 subjects. Sequences from the 5 laboratory strains retrieved from GenBank are labeled (accession numbers are in parentheses): YU.2 (M93259), BH5 (K02012), HXB2 (K03455), JRCSF (M38429), and pNL4.3 (U26942). The scale bar represents 1% nucleotide sequence divergence. The tree was constructed using the DNADIST and NEIGHBOUR programs in the PHYLIP Version 3.57c package, provided by Joseph Felsenstein, PhD.

To ensure the integrity of the sequence data obtained, a phylogenetic analysis of the 80 sequences of both protease and reverse transcriptase was performed to rule out laboratory strain and intersample contamination (Figure 2). The phylogenetic tree clearly demonstrates independent segregation of sequences from laboratory strains (HXB2, YU.2, BH5, JRCSF, pNL4.3) as well as from each other.

Phenotypic Analysis of Selected Isolates

Drug susceptibility testing was successfully performed on 67 samples using a recombinant virus assay. Phenotypic data on 9 plasma samples could not be determined because of a reduced success rate in PCR amplification of the large amplicon required to perform the assay. Additionally, plasma from 1 individual was unavailable for testing and could not be assayed because of as yet undefined technical issues. Fifty-seven (85%) of these samples had phenotypes consistent with the interpretation of the genotypic data (data not shown). Forty-nine (73%) of the 57 had a drug-sensitive phenotype with less than 3.0-fold decreased susceptibility to all tested drugs and a genotype without any known resistance mutations. Eight subjects had reduced susceptibility to selected compounds in concordance with the observed genotypes. Three of these individuals had isolated substitutions known to confer resistance to lamivudine (M184V) or NNRTIs (V179E), concurrent with the phenotypes. The presence of

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M184V was associated with high-level (>1000-fold) reduced susceptibility to lamivudine. One of the 2 viruses with an isolated substitution V119E had decreased susceptibility to the NNRTI delavirdine (4.0-fold), whereas the second revealed reduced susceptibility to all 3 NNRTIs—delavirdine (15.5-fold), nevirapine (4.9-fold), and efavirenz (6.4-fold).

Virus from 1 subject had isolated high-level reduced susceptibility to lamivudine (>1000-fold) despite the absence of M184V in the reverse transcriptase of the PCR product used for consensus sequencing. However, the M184V mutation was present in the resistance test vector pool used for the phenotypic assay. To determine whether minor viral populations could have accounted for the observed discordance, we performed limiting-dilution PCR and sequencing of 10 amplicons on the patient’s plasma. One of 10 sequences contained a genotype coding for the M184V substitution in reverse transcriptase. This individual was known to have been infected by an HIV-infected contact with intermittent adherence to a double nucleoside treatment regimen containing lamivudine.

Of the 10 cases with apparent discordance between phenotype and genotype, all had low-level but reproducible reduced susceptibility to 1 or more antiretroviral agents in the absence of known resistance-conferring genotypes. Five cases had modest (3.2- to 4.6-fold) reductions in susceptibility to nelfinavir, while in 2 cases, similar reductions in susceptibility to indinavir (3.9- and 4.9-fold) were noted. These were found in the context of genotypes lacking primary site protease mutations, including V82A, D30N, or L90M. To validate the genotypic findings, 10 to 12 replicates were selected for sequencing after limiting-dilution PCR in 5 of 7 subjects. In each case, no primary mutations were found in any of the PCR products to account for the observed phenotypes, nor did these sequences obviously differ with respect to the incidence and distribution of known polymorphisms in HIV-1 protease from those derived from patient plasma that tested sensitive in the phenotypic assay. The 3 remaining discordant cases demonstrated similarly low-level (3.2- to 3.6-fold) reduced susceptibility to delavirdine. Limiting-dilution PCR in 2 of 3 plasma samples was unrevealing and the clinical significance of this minimal reduction in susceptibility is unclear.

**Transmission of MDR Virus**

Three MDR viruses, defined by the presence of resistance-conferring genotypes and phenotypic resistance to more than 1 class of antiretrovirals, were identified (Table 3). One subject, MDR1, infected in June 1996, harbored NNRTI- and lamivudine-related resistance-conferring mutations K103N and M184V at baseline. Phenotype revealed greater than 1000-fold reduced susceptibility to lamivudine, and 79.2-fold, 93.1-fold, and 32.5-fold reduced susceptibility to nevirapine, delavirdine, and efavirenz, respectively. At the time of presentation, this subject was empirically treated with triple combination therapy, including zidovudine-lamivudine and indinavir, and demonstrated viral load rebound after a period of transient reduction lasting approximately 250 days. Virological breakthrough was associated with the emergence of protease inhibitor resistance-conferring genotypes (V82A, M46I, V32I) and phenotypic resistance to indinavir (4.1-fold) and cross-resistance to nelfinavir (3.6-fold) and ritonavir (9.0-fold) (Figure 3).

Viruses from subjects MDR2 and MDR3 revealed zidovudine resistance-conferring mutations M41L/D67N/T215Y (MDR2) and M41L/D67N/K70R/T215F/K219Q (MDR3) combined with known primary protease inhibitor resistance amino acid substitutions V82A/L90M (MDR2) and L90M (MDR3). Multidrug-resistant virus 3 also had 2 NNRTI resistance-conferring mutations at residues 179 (V to D) and 188 (Y to L).

Phenotypic data for MDR2 revealed reduced susceptibility to zidovudine (714.2-fold), lamivudine (4.3-fold), abacavir (0.5-fold), and 7.3- to 46.3-fold reductions in susceptibility to the protease inhibitors (Table 3). Multidrug-resistant virus 3 was similarly found to have reductions in susceptibility of 30.3-fold to zidovudine, 16.6-fold to saquinavir, 5.4-fold to indinavir, 8.9-fold to ritonavir, and 33.2-fold to nelfinavir. Reduced susceptibility to the 3 NNRTIs, delavirdine (36.6-fold), nevirapine (>1000-fold), and efavirenz (162.9-fold), was demonstrated.

**COMMENT**

We present the first published series to our knowledge describing the prevalence of transmitted drug-resistant HIV-1 among 80 individuals in the United States in the era of combination antiretroviral therapy, including both reverse transcriptase inhibitors and protease inhibitors. The cohort is predominantly homosexual men from large urban centers (eg, New York, Los Angeles). This cohort represents a subset of new HIV-1 infections and may not reflect the prevalence of resistance in newly acquired HIV overall. In this cohort, the overall prevalence of genotypes associated with drug resistance to any antiretroviral agent was approximately 16.3%. We believe that this represents a minimal estimate because there may be resistance-conferring mutations that have not yet been identified. Of note, of 67 phenotype assays performed, 18 (26.8%) detected a 3.0-fold or greater reduction in susceptibility to any antiretroviral agent. However, in 10 of 18 assays, low-level (3.0- to 5.0-fold) reduced susceptibility was determined, the clinical significance of which has not been established. Multidrug resistance, as defined by the presence of both resistance-conferring genotypes and resistant phenotypes (>5.0-fold) to 2 or more classes of antiretroviral drugs, was far less common, only 3.8%. Two of the 3 cases were identified in the past year. Furthermore, 5 of 7 individuals infected in 1999 harbored known resistance-conferring mutations to at least 1 antiretroviral agent. We observed extensive polymorphism in the protease gene, with high frequencies of substitutions at residues...
the interpretation of genotypes is difficult as the interpretation of resistance testing data. In highlights the difficulties in the interpretation of resistance-conferring genotypes. This by sequencing multiple products of limit transcripts in a given individual did not change during the observation period. The overall concordance between interpretations of genotypic data and phenotypic results was 85.0% in patients in whom both assays were performed. In the 10 apparently discordant cases, low-level reduced susceptibility (3.0- to 5.0-fold) to nelfinavir, indinavir, or delavirdine was observed. The careful genotypic analysis of plasma samples by sequencing multiple products of limiting-dilution PCR did not reveal known resistance-conferring genotypes. This highlights the difficulties in the interpretation of resistance testing data. Interpretation of genotypes is difficult as there are large numbers of polymorphisms in both protease and reverse transcriptase that may or may not confer some degree of drug resistance. Furthermore, although the lower limit of detection of changes in susceptibility is defined by the assay variability (2.5-fold), only carefully executed prospective trials that incorporate both genotypic and phenotypic testing will clarify the clinical significance of the observed low-level reductions in susceptibility to specific antiretroviral agents.

One subject with primary HIV-1 infection was infected with a virus resistant to lamivudine, confirmed by both genotypic and phenotypic analyses. Human immunodeficiency virus 1 RNA increased in this individual after therapy with zidovudine, lamivudine, and indinavir, associated with the emergence of resistance to indinavir as determined by both the presence of the resistance-conferring genotype and reduced susceptibility by phenotype. As this case demonstrates, baseline resistance may have significant consequences for treatment response and suggests a potential role for resistance testing in the setting of treating acute HIV-1 infection.

As previously shown,21 we observed that though rare, sexual transmission of MDR HIV is possible. Importantly, the donors were known to have high levels of circulating drug-resistant virus (plasma HIV RNA, approximately 1 million copies/mL by history). Of equal importance, episodes of high-risk sexual contact were reported by both partners of the patients we studied. The antiretroviral treatment histories of the vast majority of HIV-1–transmitting sexual partners of the patients we studied were not known.

We conclude that 4 years after the introduction of protease inhibitors and expanded options to inhibit reverse transcriptase, the prevalence of transmitted viruses with resistance-conferring genotypes to reverse transcriptase inhibitors and/or protease inhibitors in this cohort of 80 subjects is 16.3% (NRTIs, 12.5%; NNRTIs, 7.5%; protease inhibitors, 2.5%). These data support continued investigation into the use of resistance testing when treating primary HIV-1 infection. It is hypothesized that when treating acute HIV-1 infection, immediate suppression of virus replication is desired to both limit total body viral burden and preserve HIV-1–specific immune responses.1,37 Therefore, we propose that clinical trials be initiated to determine whether additional virological and immunological benefits may be derived from resistance assay–guided therapeutic regimens (multiple drugs from multiple classes initiated em-


REFERENCES


