Early Archiving and Predominance of Nonnucleoside Reverse Transcriptase Inhibitor–Resistant HIV-1 among Recently Infected Infants Born in the United States

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(See the editorial commentary by Krogstad, on pages 1393–5.)

Background. The extent to which drug-resistant human immunodeficiency virus type 1 (HIV-1) acquired through mother-to-child transmission (MTCT) or failed chemoprophylaxis populates viral reservoirs and limits responses to antiretroviral treatment in infants is unknown.

Methods. We evaluated the presence, type, and persistence of drug-resistant HIV-1 in pretreatment plasma and resting CD4+ T cells from US infants enrolled in a multicenter, open-label, phase 1/2 treatment trial of lopinavir/ritonavir (Pediatric AIDS Clinical Trials Group Protocol 1030) in young infants.

Results. Twenty-two consecutively enrolled infants initiating highly active antiretroviral therapy at a median age of 9.7 weeks and treated for up to 96 weeks were studied. Drug-resistant HIV-1 was present in 5 (23.8%) of 21 infants analyzed; 4 (80.0%) had nonnucleoside reverse transcriptase inhibitor (NNRTI)–resistant HIV-1, only 1 of whom had a history of receiving nevirapine chemoprophylaxis. All 4 infants had NNRTI-resistant variants other than the K103N mutation. The fifth infant had the M184V mutation. Drug-resistant virus was archived in the resting CD4+ T cell latent reservoir in all 5 infants.

Conclusions. The high rate, types, and early archiving of drug-resistant HIV-1 suggests that resistance testing be considered for infants, especially when an NNRTI-based regimen is planned. Furthermore, drug-resistance outcomes in infants should be an important secondary end point in MTCT trials.

In the United States and Europe, testing and treating pregnant women for HIV-1 has resulted in a dramatic decline in mother-to-child transmission (MTCT) of HIV-1, with transmission rates dropping to !2% [1, 2]. Maternal infection with drug-resistant HIV-1 variants may place the infant at risk for infection with drug-resistant virus, and use of antiretroviral drugs for prepartum maternal treatment or for intrapartum post-partum chemoprophylaxis in the infant may exacerbate the problem by selecting for drug-resistant HIV-1 in infants infected despite prophylaxis.

Drug-resistant HIV-1 arising as a consequence of infant postnatal treatment to prevent MTCT has been

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demonstrated [3]. Reported less commonly has been vertical transmission of drug-resistant HIV-1 [4–6]. Use of nevirapine (NVP) and lamivudine (3TC) as single agents or in 2-drug combinations results in selection of single-point mutations in the reverse transcriptase (RT) gene that confer high-level resistance and may preclude further use of these drugs for treatment. In US- and European-based cohort studies, zidovudine (ZDV) resistance in infants receiving ZDV prophylaxis to prevent MTCT (PMTCT) ranges from 9% to 30% [3, 7–10]. In international settings [11], nonnucleoside RT inhibitor (NNRTI) resistance has been reported at 6 weeks of age in up to 46% of HIV-1 subtype A– or D–infected Ugandan infants who received single-dose NVP (SD-NVP) [12] to PMTCT and in 87% of subtype C–infected Malawian infants [13]. Similarly, HIV-1 resistant to 3TC is observed even when used in combination with ZDV for chemoprophylaxis [14].

The extent to which drug-resistant HIV-1 populations and persists in latent viral reservoirs and the effects of early therapy on the persistence of these variants in infected infants have not been studied. In a sub-study of a phase 1/2 trial of lopinavir/ritonavir (LPVr) in infants with perinatally acquired HIV-1 infection <6 months of age, we examined the presence, type, and persistence of drug-resistant HIV-1 in latent CD4+ T cell reservoirs and their effect on treatment responses.

SUBJECTS, MATERIALS, AND METHODS

Study design and subjects. Study subjects were enrolled in the Pediatric AIDS Clinical Trials Group Protocol 1030 (PACTG P1030), an open-label, multicenter, phase 1/2 dose-finding trial of LPVr in infants with perinatally acquired HIV-1 infection <6 months of age, we examined the presence, type, and persistence of drug-resistant HIV-1 in latent CD4+ T cell reservoirs and their effect on treatment responses.

Plasma HIV-1 RNA levels. Plasma HIV-1 RNA levels were quantified using RT–polymerase chain reaction (PCR) methods (Amplicor HIV-1 Monitor Assay; Roche Diagnostics) with limits of detection of <400 and <50 copies/mL.

Culture of resting CD4+ T cells. Culture of resting CD4+ T cells was performed using a limiting dilution culture method [15, 16] adapted for use on small blood volumes (median, 2.9 mL) obtainable from infants [17].

Amplification and cloning of the HIV-1 pol gene for drug resistance. The HIV-1 pol gene (1.5 kb) was amplified and cloned from the cultured supernatant derived from coculture of purified resting CD4+ T cells and from pretreatment plasma using previously published methods [18–20]. HIV-1 RT (560 bp) was amplified using previously published methods from pre–highly active antiretroviral therapy (HAART) plasma in 2 patients in whom the 1.5-kb fragment of the pol gene was not amplifiable [21]. Commercial HIV-1 genotyping and phenotyping (Phenosense GT; Monogram Biosciences) was also performed as part of clinical care on pretreatment plasma from 1 of the study subjects.

HIV-1 sequence analysis. The pol sequences were assembled and aligned in Bioedit (version 7.0.5.2; available at: http://www.mbio.ncsu.edu/BioEdit). Substitutions that may have been induced during the PCR were corrected using Clean-Collapse (version 1.0.5; available at: http://sray.med.som.jhmi.edu/SCRoftware) [21]. HIV-1 pol was analyzed for mutations at known sites of drug resistance [22]. Because the plasma genotypes obtained before study treatment were not obtained from limiting dilution RT-PCR, only those clones that differed by sites of drug-resistance mutations were included in the phylogenetic tree. Similarly, because the viral isolates were recovered using a limiting dilution culture method, only the sequences derived from clones of viral isolates that were recovered from individual wells or those recovered from the same well but differed by drug-resistance mutations were included in the phylogenetic analysis. Phylogenetic analyses were performed using HyPhy (version 0.99; available at: http://www.hyphy.org) [23]. Analysis of recombination was performed using SimPlot (version 3.5.1; available at: http://sray.med.som.jhmi.edu/SCRoftware) [24]. The sequences were submitted to Genbank (accession numbers EF066742–EF067312).

Statistical analysis. Fisher’s exact test was used to evaluate differences in response frequencies at week 24 between drug-resistant and drug-sensitive groups. Wilcoxon’s rank-sum test was used to compare pretreatment viral load, virologic response rates, and CD4+ and CD8+ T cell levels between drug-resistant and drug-sensitive groups.

RESULTS

Patient characteristics. Twenty-two US-born subjects enrolled in P1030 had blood samples available for analysis; an additional infant was not included because he had not reached week 24 of study treatment. Thirteen of the 22 infants received postnatal chemoprophylaxis with ZDV; 3 received ZDV and NVP; 1 received ZDV, 3TC, and NVP; and 5 received no che-
moprophylaxis. The median duration of ZDV chemoprophylaxis was 4.1 (range, 2.3–8.0) weeks. Antiretroviral treatment history during pregnancy was available for 14 mothers: 5 of 14 received combination antiretroviral therapy, and 2 received intravenous ZDV during labor only (table 1). Maternal country of origin was available on 12 infants who were coenrolled in PACTG P219C: 7 mothers were US born, 1 was from Puerto Rico, 1 from Honduras, 1 from Mexico, and 2 from West Africa. The median plasma HIV-1 RNA level in infants before HAART was 5.7 (range, 3.7–6.9) log copies/mL (table 1). Study treatment was initiated at a median age of 9.6 (range, 3.6–25.7) weeks.

**Extent and types of primary infection with drug-resistant HIV-1.** HIV-1 genotyping on pre-HAART plasma was successful in 21 subjects (95.5%) of 22; in subject 16, pre-HAART amplification was not successful due to insufficient plasma volume (<140 mL). The median age of the subjects at baseline analysis of plasma was 9.6 (range, 3.6–25.7) weeks.

A total of 167 clones from 45 independent RT-PCRs performed on plasma were analyzed. A median of 2 RT-PCRs were performed on pre-HAART plasma, and the median number of pre-HAART clones analyzed per patient was 7. Phylogenetic analysis confirmed the patient specificity of the pol sequences amplified from plasma (figure 1). Most of the subjects (19/21) had subtype B infection; subjects 10 and 18 were infected with subtype A/G recombinant virus (figure 1). Sequences from subject 10 were most closely related to CRF02_AG reference sequences, whereas sequences from subject 18 were consistent with additional recombination between CRF02_AG and subtype G (data not shown) as described in the Los Alamos HIV sequence database annotation for Ghanian isolate 94GH09 (GenBank accession number AF212289). Infection with subtype A/G recombinant virus was consistent with maternal countries of origin in West Africa. Five (23.8%) of 21 infants had drug-resistant virus present in pretreatment plasma (figure 1 and table 1). The remaining 16 subjects (76.2%) were infected with wild-type virus with no pol gene mutations associated with drug resistance.

A total of 60 cDNA clones from 20 RT-PCRs were analyzed from the 5 subjects with drug-resistant HIV-1 (figure 2). NNRTI-resistant variants were present in pretreatment plasma of 4 (80%) of these 5 subjects. Maternal antiretroviral treatment during pregnancy was available for 4 of the 5 infants infected with drug-resistant HIV-1 and for 3 of the 4 infants infected with NNRTI-resistant variants. Information on the drugs used for chemoprophylaxis was available for all 5 infants. Information on maternal viral loads and genotypes was not available.

Only 1 mother of the infants infected with NNRTI-resistant variants (the mother of subject 9) is known to have taken a NVP-containing regimen during pregnancy. This infant was infected at baseline with HIV-1 resistant to multiple drug classes. The NNRTI mutations included variants having V106A or Y188C linked to nucleoside RT inhibitor (NRTI) mutations M41L, M184V, and T215Y (figure 2).

Infant 3 received chemoprophylaxis with NVP. In this subject, 2 doses of NVP in addition to 6 weeks of ZDV and 1 week of 3TC were used. The mutations detected were K101P and K103S in RT. These mutations have not been reported to occur with NVP chemoprophylaxis but are known to confer high-level resistance to NVP. Clonal analysis of pretreatment plasma obtained at 6 weeks of age showed that all 24 clones derived from 5 independent RT-PCRs (figure 2) had the K101P and the K103S mutations. In addition, 4 (17%) of the 24 clones had M184V linked with K101P and K103S. A commercial genotype done as part of clinical care on plasma at 3 weeks of age and before study treatment showed the K101P and K103S mutations. In addition, 4 (17%) of the 24 clones had M184V substitutions. The phenotype on this sample showed >100-fold resistance to delavirdine, efavirenz, and NVP. A repeat genotype done for clinical care at 4 weeks of age showed persistence of K101P and K103S, but M184V was no longer detectable. Phenotypic testing confirmed high-level resistance (defined as greater than the maximal fold change in resistance) to NNRTIs on the second clinical sample, but phenotypic resistance to 3TC was not detected. These findings are consistent with the relatively low frequency of this variant in the plasma analyzed at 6 weeks of age using clonal sequencing methods.

In the remaining 2 infants (4 and 7) infected with NNRTI-resistant variants, NVP-containing regimens were not used during pregnancy or for chemoprophylaxis. Both of these infants had a mixture of NNRTI-resistant variants at baseline. For patient 4, all 7 clones derived from 2 independent RT-PCRs performed on pre-HAART plasma had the G190A mutation. In patient 7, V106I (6/7 clones) or V106I linked with Y188H (1/7 clones) was present in pre-HAART plasma (table 1 and figure 2).

Infant 1 was infected with M184V, which confers resistance to 3TC. All clones analyzed from pre-HAART plasma from this subject contained this mutation. This infant’s mother received treatment with ZDV and 3TC during pregnancy before addition of the protease inhibitors LPVr and saquinavir. In this infant, both ZDV and NVP were used for chemoprophylaxis.

**Extent of sampling and recovery of infectious HIV-1 from the resting CD4+ T cell population.** Blood samples were obtained longitudinally on 91% of 22 subjects for evaluation of persistence and development of drug resistance in the resting CD4+ T cell reservoir. Sixteen infants were evaluated at the first time point (median, 5.6 [range, 4.8–7.5] months on study treatment) and an overlapping group of 18 at the second time point. All 9 infants who remained on study through 96 weeks were analyzed at the last study point (median, 95.9 [range, 93.0–103.1] weeks). Samples were not received on 6 infants at the first time point, and 4 infants were not studied at the second
Table 1. Patient characteristics and virologic and antiretroviral treatment data.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Maternal antiretroviral treatment during pregnancy</th>
<th>Chemoprophylactic regimen</th>
<th>Duration of chemoprophylaxis, weeks</th>
<th>Age at start of HAART, weeks</th>
<th>Viral load, log10 HIV-1 RNA copies/mL</th>
<th>Antiretroviral treatment regimen</th>
<th>Pre-HAART HIV-1 pol plasma genotypes</th>
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NOTE. 3TC, lamivudine; ABC, abacavir; AZT, zidovudine; D4T, stavudine; DDI, didanosine; IDV, indinavir; LPV, lopinavir; LPV, lopinavir/ritonavir; na, not applicable; NA, not available.

a Coenrolled in Pediatric AIDS Clinical Trials Group Protocol 219C.
Figure 1. Phylogenetic analysis of HIV-1 reverse transcriptase sequences derived from pre–highly active antiretroviral therapy (HAART) plasma and latent reservoir (LR) clones, showing infant-specific clustering and the presence of non–subtype B sequences. Triangles and circles represent plasma and replication-competent LR-derived sequences, respectively, with colors indicating visit no. as shown in the key. Symbols with strikes represent sequences that contained drug-resistance mutations. Bootstrap support (proportion of 1000 permuted trees) is indicated with asterisks.
Figure 2. Analysis of cDNA clones from 20 reverse-transcriptase polymerase chain reactions (RT-PCRs) from the 5 subjects with drug-resistant HIV-1. Nos. in the top row indicate RT codons associated with canonical drug resistance to RT inhibitors. 3TC, lamivudine; ABC, abacavir; AZT, zidovudine; D4T, stavudine; DDI, didanosine; HAART, highly active antiretroviral therapy; LPVr, lopinavir/ritonavir; NNRTI, nonnucleoside RT inhibitor; NRTI, nucleoside RT inhibitor; NVP, nevirapine.
time point because of death unrelated to treatment (1), treatment discontinuation (2), and not yet reaching the 48-week study visit (1).

Thirteen (65%) of 20 subjects had undetectable HIV-1 RNA (<400 copies/mL) in plasma at the time of first analysis of the reservoir, 6 had a median decrease in viral load of 2.44 log_{10} copies/mL from baseline, and 1 infant had no change in viral load from baseline (table 1). Eight (89%) of 9 were studied at the final time point. Detailed virologic and pharmacologic data on the cohort will be reported elsewhere (E.G.C. et al., unpublished data). Replication-competent virus was recovered from the resting CD4+ T cell latent reservoir in 19 (95%) of 20 evaluable subjects. In the only infant from whom virus was not recovered, only 1 sample was available, and it contained insufficient cells (<2.25 million cells) for culture. From these 19 subjects, 132 independent viral isolates (median, 4.5 [range, 1–30] viral isolates/subject) were genotyped for this study.

**Extent of persistence of drug-resistant HIV-1 variants acquired during MTCT in early-treated infants.** Wild-type virus populated and persisted in the resting CD4+ T cell reservoir in 15 of 16 infants with wild-type HIV-1 at baseline. In 1 infant with wild-type HIV-1 at baseline, virus was not recovered at 24 weeks; however, 3 isolates recovered at 48 weeks all had the M184V mutation. In this patient, the detection of drug-resistant HIV-1 in the reservoir was temporally associated with poor adherence and rebound viremia after an initial period of undetectable viral load, although infection with 3TC-resistant HIV-1 cannot be fully excluded.

Figure 2 summarizes the drug-resistance profiles in HIV-1 RT for the 56 viral isolates cultured from the 5 subjects infected with drug-resistant virus and compared with pre-HAART plasma genotypes. All infants with drug-resistant HIV-1 before HAART maintained a viral reservoir populated with drug-resistant variants identical to those present before treatment. For subject 3, 2 viral isolates were recovered at 32 weeks of HAART, and all 3 clones derived from the 2 isolates had the K101P and K103S mutations. One of the clones had the K101P and K103S substitutions in association with the M184V mutation seen in pretreatment plasma. Likewise, for subject 4, all 26 viral isolates recovered at 3 different time points over 96 weeks of study treatment harbored the G190A mutation seen at baseline. This subject had a baseline plasma viral load of 6.9 log_{10} HIV-1 RNA copies/mL of plasma before therapy and had a slow decay in plasma viremia on HAART. The subject achieved a viral load of <2.6 log_{10} HIV-1 RNA copies/mL after 51 weeks of HAART. At week 132 of follow-up, plasma virus remained undetectable on the primary HAART regimen. Similarly, for subject 7, the 7 viral isolates cultured at 96 weeks of study all contained the V106I mutation that was dominant at baseline. In infant 9, infected with multiclass drug-resistant HIV-1, viral cultures (12 isolates) at all 3 time points consisted of mixtures of viral variants (4 genotypes) with the same mutation profiles as those predating HAART. In this subject, all the isolates had the M41L and T215Y mutations, 7 of 12 isolates also had the V106A mutation, 3 of 12 also had V106A and M184V, 2 of 12 also had the Y188C, and 1 of 12 had the M184V (Figure 2). All variants were also detected at baseline in plasma from this subject, who initially did well on HAART with sustained suppression of viral load to <2.6 log_{10} HIV-1 RNA copies/mL between weeks 12 and 20 of HAART before rebounding at week 24 of HAART because of nonadherence. With improved adherence, the viral load suppressed to <400 copies/mL on the primary HAART regimen.

Importantly, these NNRTI mutations persisted despite the fact that none of these infants received NNRTI-based HAART. Similarly, in subject 1 who was infected with the M184V 3TC-resistant mutant, all 5 viral isolates cultured from resting CD4+ T cells at 24 and 48 weeks of treatment had only the M184V mutation (Figure 2). Although this subject’s HAART regimen contained 3TC, the patient achieved an undetectable viral load by 12.1 weeks of HAART and remained suppressed for the study duration of 96 weeks.

**Baseline viral load, CD4+ and CD8+ T cell levels, and treatment response.** Associations between the effects of infection with drug-resistant versus wild-type HIV-1 on pretreatment viral load and response to primary HAART were assessed. Infants were categorized as “nonresponders” if plasma HIV RNA load was >400 copies/mL at week 24 and confirmed after week 24. Among 20 participants who had both pre-HAART genotype information and had reached week 24, the percentage of children considered responders at week 24 was 40% (2/5) for the drug-resistant group and 80% (12/15) for the drug-sensitive group. There was no significant difference in treatment response between groups (P = .13), but the power to detect clinically relevant differences was limited by the small sample size. Baseline median viral load was similar at 5.6 versus 5.8 log_{10} HIV-1 RNA copies/mL for the drug-resistant and drug-sensitive groups, respectively (P = 1.0). Furthermore, there was no difference in median baseline CD4+ T cell percentage (34% vs. 38%) and CD8+ T cell percentage (20% vs. 23%) between the 2 groups (P = .33 and P = .77, respectively).

**DISCUSSION**

Almost a quarter of a cohort of recently infected US-born infants were infected with drug-resistant HIV-1, which was predominantly resistant to NNRTIs, and, in all infants, the drug-resistant variants were archived in resting CD4+ T cells within the first 6 months of life. The increase in primary infection with NNRTI-resistant HIV-1 is reflective of trends of primary infection with drug-resistant HIV-1 in adults in the United States and Europe [25–27] and in treatment-naive pregnant women [28]. Although the existing literature on drug-resistant
HIV-1 in infants emphasizes resistance arising because of chemoprophylaxis to PMTCT, 4 of the 5 infants in this study did not receive antiretroviral drugs that would select for the drug-resistant variants with which they were infected. An increase in prevalence of infection with drug-resistant HIV-1 and predominantly NNRTI resistance was recently reported among HIV-1–infected infants in New York State [29]. This implies that primary infection with drug-resistant HIV-1 and predominantly with NNRTI resistance is more common than previously reported.

In our cohort, the NNRTI-resistant variants detected were heterogeneous and included mutations other than K103N commonly seen with NNRTI-treatment failure. Only 1 subject had a substitution at amino position 103 in RT, and this was a K103S substitution that has been reported to occur in <0.5% of clinical isolates and to confer >10-fold decreased susceptibility to NNRTI [30]. The combination of K103S with K101P substitutions in RT conferred a 100-fold decreased susceptibility to all the NNRTIs by phenotypic testing. In this infant, because maternal genotypes were not available, selection of these mutations by postnatal chemoprophylaxis with NVP and 3TC cannot be excluded. Although this study is limited by the lack of availability of complete maternal antiretroviral treatment histories and genotypes, collectively, these findings highlight the transmission of NNRTI drug-resistance variants, the selection of variants other than the common K103N variant, and the potential for these mutants to be missed when more sensitive, single-point mutation assays are used for monitoring transmission of drug-resistant HIV-1 [31–34].

Infection with drug-resistant HIV-1 in infants has important implications for therapy. In untreated HIV-1–infected adults, both the long-term persistence of plasma viremia with transmitted drug-resistant HIV-1 [35, 36] and decline in detection of these transmitted variants by replacement with wild-type HIV-1 have been reported [37]. The long-term persistence in plasma of NNRTI-resistant HIV-1 variants acquired during MTCT has been examined in untreated infants in resource-poor settings. These studies have shown that, although in some infants resistant variants persist for up to 18 months in plasma, in most infants, these drug-resistant variants become undetectable in plasma even when tested by ultrasensitive genotyping methods [31]. The degree to which drug-resistant HIV-1 acquired during MTCT or during postnatal prophylaxis populates and persists in replication-competent latent viral reservoirs, and whether delayed initiation of antiretroviral therapy in infants alters the extent to which the resting CD4+ T cell reservoir becomes populated with drug-resistant virus was not known.

We show that drug-resistant HIV-1 in perinatally infected US infants can fully populate the resting CD4+ T cell reservoir early in the course of infection and persists in replication-competent forms for years, even in the absence of drug-selective pressure (i.e., when HAART does not include the drug[s] to which the variants are resistant). Importantly, in the context of the parent phase 1/2 clinical trial of LPVr, the observed drug-resistance mutations in RT did not preclude suppression of HIV-1 replication with LPV-based HAART. Furthermore, none of the infants developed new substitutions in protease at sites that contribute to resistance during the study period (data not shown). The long-term impact on HAART effectiveness, however, will require more study.

The types of drug-resistance mutations and the high level of resistance they conferred found in this study suggest that primary infection with drug-resistant HIV-1 occurs in a significant proportion of infants and that genotypic drug-resistance testing for children initiating therapy in the first months of life likely is warranted. This may be especially important to consider when NNRTI-based HAART is used for the treatment of HIV-infected infants. Furthermore, given that SD-NVP is the cornerstone of efforts to PMTCT in resource-limited settings and that current pediatric and adult World Health Organization treatment recommendations and practice in resource-limited settings depend on NNRTI-based HAART, longitudinal studies are needed to monitor the frequency and nature of drug-resistant HIV-1 in primary infection in infants and its impact on clinical outcomes.

References


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