Evaluation of Lymph Node Virus Burden in Human Immunodeficiency Virus–Infected Patients Receiving Efavirenz-Based Protease Inhibitor–Sparing Highly Active Antiretroviral Therapy

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Although efavirenz-containing regimens effectively suppress plasma levels of human immunodeficiency virus (HIV) RNA, it is now clear that undetectable plasma viremia may not reflect a lack of viral replication. Because lymphoid tissue is an active site of HIV replication, the lymph node virus burden was analyzed in persons who received highly active antiretroviral therapy (HAART) containing either efavirenz or a protease inhibitor (PI). Testing with in situ hybridization revealed no detectable follicular dendritic cell–associated HIV RNA in either group, and only 2 of 8 persons in the efavirenz group and 1 of 4 in the PI group had detectable RNA in lymph node mononuclear cells (LNMC) when tested by use of nucleic acid sequence–based amplification. Low levels of replication-competent HIV were identified in both groups by use of quantitative coculture assays. There was no evidence of development of resistance to either regimen in virus isolated from LNMC. These data support the use of efavirenz as an alternative to a PI in initial HAART regimens.

The use of highly active antiretroviral therapy (HAART) has resulted in a considerable improvement in mortality and a decrease in adverse events for persons infected with human immunodeficiency virus (HIV) [1]. The US Food and Drug Administration (FDA) has approved 16 antiretroviral drugs for the treatment of HIV, including nucleoside analogue reverse-transcriptase inhibitors (NRTIs), nonnucleoside reverse-transcriptase inhibitors (NNRTIs), and protease inhibitors (PIs) [1]. The diversity of medications has led to difficult decisions for physicians and patients who must choose a regimen that takes into account efficacy, toxicity, and effect on lifestyle. Treatment guidelines have been developed to assist in the selection of the best therapeutic regimen for individual patients.

Current guidelines recommend 2 major options for initial therapy: 2 NRTIs combined with either a PI or the NNRTI efavirenz [1]. It has become evident that, despite the efficacy of PIs, considerable toxicity is associated with their extended use [2–5]. This has heightened interest in the initial use of PI-sparing regimens for the treatment of HIV disease. In this regard, the use of efavirenz as an alternative to a PI is supported by recent data demonstrating comparable suppression of plasma virus levels at 48 weeks of follow-up in PI-containing versus efavirenz-containing regimens [6, 7]. However, measurements of plasma viremia may not be the best indicator of suppression of viral replication by an antiretroviral regimen. Recent studies demonstrate that HIV-infected persons receiving HAART for extended periods have evidence of viral replication in the absence of detectable plasma viremia [8–10], although they have no genetic evidence of resistance to therapy [10].

Lymph nodes are a site of active viral replication during periods of diminished or undetectable plasma viremia [11–18]. HIV RNA has been identified in the follicular dendritic cell (FDC) network and lymph node mononuclear cells (LNMC) from persons receiving combination NRTI therapy, despite undetectable plasma viremia [12, 15, 18]. In contrast, persons receiving HAART that includes a PI have either low levels or levels below detectability of HIV RNA in their lymph nodes [11–17, 19]. In some studies, virus isolated from LNMC of persons receiving combination NRTIs showed evidence of genetic mutations to the NRTIs in the treatment regimens, indicating active viral replication and the potential for eventual
failure of the therapy [15, 18, 19]. Persons receiving effective PI-containing combination therapy did not have evidence of genetic mutations associated with resistance to therapy [15, 19]. These data argue for the superiority of PI-containing regimens over combination NRTIs in suppressing viral replication in lymphoid tissue and in avoiding the emergence of resistance-conferring mutations.

Although efavirenz-containing regimens suppressed plasma virus as efficiently as PI-containing regimens for 48 weeks [6, 7], it is uncertain whether the combination of efavirenz and NRTIs can suppress viral replication in lymphoid tissue with an effectiveness similar to that of PI-containing regimens. Thus, we obtained lymph nodes from 9 persons who had been naive to antiretroviral therapy before being treated effectively with stavudine, lamivudine, and efavirenz for 7–8 months. As a control, lymph nodes were analyzed from a smaller group of subjects receiving 2 NRTIs and a PI, as shown in table 1. All persons in both groups achieved and maintained an undetectable plasma virus load of $<50$ copies/mL, as determined by testing with ultrasensitive bDNA or reverse-transcriptase (RT) polymerase chain reaction (PCR). At the time of lymph node biopsy (see below), virus load was determined by use of bDNA, and CD4 T cell counts were determined by use of flow cytometry, to analyze peripheral blood samples.

### Materials and Methods

#### Patients

Participants were recruited from their primary care physicians for this cross-sectional study. All patients were antiretroviral naive at initiation of their HAART regimens. Patients did not have other immunologic diseases or a history of opportunistic infections.

#### Drug regimens and assays for plasma virus

The PI-sparing group consisted of 9 persons who received stavudine, lamivudine, and efavirenz, and the PI-containing group comprised 4 subjects who received 2 NRTIs and a PI, as shown in table 1. All subjects achieved and maintained an undetectable plasma virus load of $\leq 50$ copies/mL, as determined by testing with ultrasensitive bDNA or reverse-transcriptase (RT) polymerase chain reaction (PCR). At the time of lymph node biopsy (see below), virus load was determined by use of bDNA, and CD4 T cell counts were determined by use of flow cytometry, to analyze peripheral blood samples.

#### Lymph node biopsy

Excisional inguinal lymph node biopsies were performed after 7–8 months of therapy, according to a protocol approved by the institutional review board of the National Institute of Allergy and Infectious Diseases. The middle one-third of each biopsy specimen was immediately placed in ParaFlex (MoLecular Histology Laboratories, Rockville, MD) for in situ hybridization and immunohistochemical analyses, as described below. LNMC were isolated from the remaining tissue by teasing out cells, followed by centrifugation with ficoll-hypaque.

#### In situ hybridization and immunohistochemistry

In situ hybridization was performed by a standard procedure [20] in which paraffin-embedded tissues were sectioned at 6 μm and hybridized with 35S-labeled probes representing 90% of the HIV genome. The
slides were dipped in Kodak NTB emulsion and exposed for 4 days. Immunohistochemistry was done with primary antibodies and a staining kit from Dako (Carpentaria, CA). The secondary antibodies were revealed by use of horseradish peroxidase 3,3-diaminobenzidine.

**Quantification of cell-associated HIV RNA by NASBA.** We lysed 10^6 LNMC with 9 mL of buffer containing guanidine thiocyanate and Triton X-100 and froze the LNMC at −70°C. HIV RNA was quantified with an NASBA, as described elsewhere [21]. In brief, first-strand synthesis of cDNA was accomplished by primer 1 (containing the T7-RNA polymerase promoter sequence) and avian myeloblastosis virus (AMV) RT. The RNA of the heteroduplex (RNA-DNA) was degraded by RNase H; the synthesis of the second-strand cDNA was performed by use of primer 2 and AMV-RT, and RNA transcripts were produced by T7-RNA polymerase. Various concentrations of synthetic RNAs derived from the cDNA sequence of HIV-1 were used as internal standards in the reaction. Signal was detected after hybridization using ruthenium-labeled probe and was quantified by measuring chemiluminescence. The primer pairs and probes have been described elsewhere [21].

**Quantitative coculture assays.** CD4 T cells were isolated from LNMC by use of a column-based purification technique (StemCell Technologies, Vancouver), as described elsewhere [22]. The purity of the cell suspensions was 95%–99%. To determine the frequency of CD4 T cells from infected patients carrying replication-competent HIV, we performed standard dilutional quantitative micrococulture assays, as described elsewhere [22]. In some experiments, we performed high-input quantitative coculture assays, in which multiple wells containing 10^6 cells were subjected to activation to determine the frequency of cells carrying replication-competent HIV, as described elsewhere [22].

**Phenotypic and genotypic resistance testing.** Phenotypic analysis of virus isolates was performed by use of the recombinant virus-assay approach (Antivirogram; Virco, Mechelen, Belgium), as described by Hertogs et al. [23], with modifications described by Pauwels et al. [24]. In brief, protease (PR)– and RT-coding sequences were amplified from patient-derived viral RNA with HIV–1–specific primers. After homologous recombination of ampiclons into a PR-RT–deleted proviral clone, the resulting recombinant viruses were harvested, titrated, and used for in vitro testing of susceptibility to antiretroviral drugs. The results of this analysis are expressed as fold-resistance values, reflecting the fold increase in mean IC_{50} (µM) of a particular drug when tested with patient-derived recombinant virus isolates, relative to the mean IC_{50} (µM) obtained when the same drug was tested with a reference wild-type virus isolate (IIIB/LAI).

Genotypic analysis was performed by automated population-based full-sequence analysis. Results of the genotypic analysis are reported as amino acid changes at positions along the RT and PR genes, compared with the wild-type (HXB2) reference sequence. Cluster analysis of individual sequences allowed detection of mutational patterns in the database containing the genetic sequences of the clinical isolates and of linkage with the corresponding resistance profiles of similar isolates.

**Results**

**Effect of antiretroviral therapy.** All subjects achieved a plasma virus load of <50 copies/mL within 16 weeks of beginning therapy, and this effect was maintained until the time of lymph node biopsy. As illustrated in table 1, group 1 comprised patients 1–9, who were treated with stavudine, lamivudine, and efavirenz. Group 2 comprised patients 10–13, who were treated with 2 NRTIs and a PI. Virus loads were determined before biopsy by use of ultrasensitive bDNA (patient 10) or by use of RT-PCR (patients 1–9, 11–13). At the time of lymph node biopsy, all patients had a plasma virus load of <50 copies/mL, as determined by use of bDNA (data not shown).

**In situ hybridization for HIV RNA.** Multiple sections from all lymph nodes were reviewed. Representative nodes from a subject in each group are shown in figure 1. Consistent with previous reports [14–17], subjects treated with a PI-containing regimen had no FDC-associated HIV RNA when tested by use of in situ hybridization (figure 1; data not shown). Patient 2 had a single mononuclear cell that expressed HIV RNA in 1 section (data not shown). Of note, in contrast to a previous report of subjects who were receiving effective combination NRTIs [15], lymph nodes from subjects in group 1 who were receiving an efavirenz-based PI-sparing regimen had no HIV RNA in the FDC network (figure 1; data not shown). A single mononuclear cell that expressed HIV RNA was identified in a lymph node section from patient 14 (data not shown). Thus, despite the higher pretherapy plasma virus load in group 1 (table 1), in situ hybridization showed no substantial HIV RNA in lymph nodes from subjects in either cohort who were effectively treated for 7–8 months. With results similar to those reported elsewhere [17], our immunohistochemical analysis of both groups revealed residual p24 antigen in the FDC network and rare association with mononuclear cells (data not shown).

**HIV RNA quantification by NASBA.** To further evaluate the lymph node virus burden in persons treated with an efavirenz-based HAART regimen, NASBA was performed on 10^6 LNMC from 12 participants to quantify cell-associated HIV RNA (assay limit, 100 copies/unit volume). In total, 3 subjects had quantifiable HIV RNA: 2 of 8 in group 1 and 1 of 4 in group 2 (figure 2). The latter finding is consistent with previous reports of low-level or undetectable LNMC HIV RNA shown by RT-PCR in persons receiving PI-based HAART [12–14, 17].

**Quantitation of lymph node–derived CD4 T cells containing replication-competent HIV.** We performed coculture experiments to determine the frequency of CD4 T cells carrying replication-competent HIV. As shown in figure 3, the majority of persons in each group (5 of 8 in group 1 and 2 of 3 in group 2) had <1 infectious unit per million cells (IUPM). The median IUPM for group 1 was 0.7 (range, 0.3–32.6), and the median IUPM for group 2 was 0.4 (range, 0.2–3.2). Other investigators have reported low or undetectable levels of infectious virus in coculture assays of LNMC from subjects receiving an effective PI-containing regimen [15, 17, 25]. However, there have been no reported evaluations of replication-competent HIV from...
Figure 1. Results of in situ hybridization. A, Lymph node biopsy specimen from untreated control (darkfield; original magnification, ×7). Large amounts of HIV RNA are seen as silver grains in germinal centers (GC). B and C, Lymph node biopsy specimens (darkfields; original magnification, ×7) from patient 2 (receiving an efavirenz-containing regimen) and patient 11 (receiving a PI-containing regimen), respectively, with no HIV RNA detected by in situ hybridization.

LNMC of persons treated with an efavirenz-based PI-sparing regimen. Of note, the patients with the highest NASBA levels—patient 7 (2500 copies/10⁶ cells) and patient 6 (49,000 copies/10⁶ cells)—also had the highest IUPM in LNMC CD4 T cells (8.33 and 32.55 IUPM, respectively; figures 2 and 3).

Phenotypic and genotypic resistance testing. HIV in supernatants from coculture experiments was analyzed by genotypic and phenotyping methods. In genotypic analyses, the HIV PR-RT genes from 4 subjects contained changes that may be associated with reduced susceptibility to the NNRTIs nevirapine, delavirdine, and efavirenz [26, 27] (table 2). Of note, only 2 of the 4 subjects were in the PI-sparing cohort. Thus, without prior exposure to NNRTIs, virus isolated from 2 of 3 persons receiving PI-containing regimens had genetic mutations consistent with the possibility of reduced susceptibility to all 3 FDA-approved NNRTIs. No major or primary genetic changes associated with reduced susceptibility to NRTIs or PIs were detected. This is consistent with previously published studies that did not find genetic mutations to NRTIs or PIs in HIV isolated from LNMC of persons effectively treated with a PI-containing regimen [15, 19].

To evaluate the relevance of these genotypic alterations, phenotypic studies were performed. None of the patients whose virus had genotypic patterns suggesting decreased susceptibility to NNRTIs had reduced susceptibility to efavirenz when tested by phenotypic assay (table 2). However, of the 4 subjects whose virus manifested these genotypic changes, 2 of 2 persons receiving a PI and 1 of 2 receiving efavirenz displayed reduced susceptibility to nevirapine, delavirdine, or both on phenotypic analysis. In addition, when tested by the phenotypic assay, 4 subjects receiving an efavirenz-based regimen had slightly decreased susceptibility to nevirapine, delavirdine, or both without genetic alterations (table 2). One person receiving an efavirenz-based regimen had slightly diminished phenotypic sensitivity to nevirnavir, and 1 person receiving a PI had diminished phenotypic sensitivity to stavudine and zalcitabine in the absence of genotypic changes (table 2).

Discussion

Given the large number of antiretroviral agents available for the treatment of HIV infection, the selection of an appropriate
combination of drugs for individual patients is often a difficult process that has been facilitated by the availability of treatment guidelines [1]. Until recently, these guidelines have recommended 2 NRTIs and a PI as the standard initial HAART regimen for HIV-infected persons who require therapy [1]. However, given the increasing frequency and severity of toxic side effects associated with the PIs, including renal stones or sludging [2], hyperglycemia, hypertriglyceridemia, hypercholesterolemia, cardiovascular disease [3], lipodystrophy [4], and compliance issues [5], there has been a growing interest in the use of PI-sparing regimens as initial therapy for HIV disease. In fact, treatment guidelines were recently modified to include the NNRTI efavirenz as an acceptable substitute for a PI in primary therapy [1]. This recommendation is supported by the durability of suppression of plasma viremia at 48 weeks of follow-up in patients receiving PI-containing versus efavirenz-containing regimens [6, 7]. However, a lack of detectable plasma viremia does not necessarily reflect complete suppression of viral replication [8–10], and it is well established that lymphoid tissue serves as an important reservoir for HIV [28, 29]. Furthermore, viral RNA can be detected in the FDC network and LNMC of infected persons who are receiving combination NRTIs, investigators could culture virus from patient LNMC [15, 18]. By use of a highly sensitive technique with large numbers of cells that were enriched for the CD4 pool, we were able to culture virus from both the PI-sparing and PI-containing groups. However, the levels were relatively low in both groups (figure 3).

The analysis of genotypic and phenotypic resistance, although limited, is of interest; however, the clinical significance of these findings is unclear. Virus isolates from persons naive to antiretroviral drugs may show evidence of reduced susceptibility to NNRTIs [26, 27]. Because 2 of 4 patients in the present study showed genetic evidence for diminished susceptibility to NNRTIs although they had never received an NNRTI, it is unlikely that therapy affected the presence of the genetic alterations. In addition, all subjects in group 1 (PI-sparing regimens) achieved and maintained an undetectable plasma virus load, with no evidence of significant viral replication in their lymph nodes for the 7–8 months of the study. At the time of writing, most of the group 1 patients had been receiving therapy for 14 months and had maintained an undetectable virus load (<20 copies/mL, detected by use of RT-PCR; data not shown). Furthermore, patients 6 and 7, who had detectable HIV RNA by NASBA and the highest IUPM levels were relatively low in both groups (figure 3).

The divergence, in some individuals, between the phenotypic and genotypic parameters of reduced susceptibility is not sur-

Table 2. Genotypic and phenotypic drug susceptibility.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Genotypic resistance</th>
<th>Phenotypic resistance, fold-increase in IC50 from WT</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>WT</td>
<td>Nevirapine, 4.9</td>
</tr>
<tr>
<td>3</td>
<td>WT</td>
<td>Delavirdine, 4.9</td>
</tr>
<tr>
<td>4</td>
<td>WT</td>
<td>All ≤4</td>
</tr>
<tr>
<td>5</td>
<td>AsS</td>
<td>Nevirapine, 13.5; delavirdine, 7.2</td>
</tr>
<tr>
<td>6</td>
<td>WT</td>
<td>Delavirdine, 8.8</td>
</tr>
<tr>
<td>7</td>
<td>WT</td>
<td>All ≤4</td>
</tr>
<tr>
<td>8</td>
<td>K101Q</td>
<td>All ≤4</td>
</tr>
<tr>
<td>9</td>
<td>WT</td>
<td>Nevirapine, 4; delavirdine, 5.7; nelfinavir, 4.1</td>
</tr>
<tr>
<td>10</td>
<td>AsS</td>
<td>Nevirapine, 6.2</td>
</tr>
<tr>
<td>11</td>
<td>None</td>
<td>All ≤4</td>
</tr>
<tr>
<td>12</td>
<td>AsS</td>
<td>Nevirapine, 7.5; delavirdine, 9.9; zalcitabine, 4.2; stavudine, 6.4</td>
</tr>
<tr>
<td>13</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

NOTE. For phenotypic assays, a score of ≤4 was considered sensitive, 4–10 was considered possibly resistant, and >10 was considered resistant. The drugs tested were zidovudine, lamivudine, didanosine, zalcitabine, stavudine, abacavir, adefovir, nevirapine, delavirdine, efavirenz, indinavir, ritonavir, nelfinavir, and saquinavir. ND, not done; WT, wild-type; AsS, alanine to serine at position 98; K101Q, lysine to glutamine at position 101.
prising. Given the mutations and the low levels of phenotypically reduced susceptibility, we would not have predicted clinically significant resistance. Further studies are required to evaluate these issues. The results of our investigation in virus isolated from LNMC show no evidence that resistance to therapy developed as a consequence of either regimen.

In summary, although a direct comparison of the 2 groups is difficult because of the difference in sample sizes, the present study is, to our knowledge, the first that examines lymph node virus burden in patients receiving an efavirenz-based PI-sparing regimen. The findings in the efavirenz-treated group are similar to those in the smaller group of patients receiving a PI in the present study and to the substantial body of literature on lymph node virus burden in persons receiving effective PI-containing HAART regimens [11–18, 25]. These data provide further virologic support for the use of efavirenz as a reasonable alternative to a PI in an initial HAART regimen for the treatment of HIV disease. However, it should be pointed out that, in addition to the peripheral lymph nodes, other potential reservoirs of HIV exist, including the reproductive tract, intestinal lymphoid tissues, brain, and bone marrow [30, 31]; the relative effects of these 2 types of regimens on these other potential reservoirs is unclear at present. Ongoing randomized clinical trials comparing PI-sparing and PI-containing regimens are necessary to determine the ultimate success of these therapeutic approaches.

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References


