Altered Viral Fitness of HIV-1 Following Failure of Protease Inhibitor-Based Therapy

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Summary: HIV-1 isolated from patients with improved CD4+ T-cell counts despite virologic failure on a nucleoside reverse transcriptase inhibitor (NRTI) and protease inhibitor (PI)-containing regimen were characterized. Five paired virus isolates from patients before and after zidovudine, lamivudine, and ritonavir treatment were tested. Human peripheral blood leukocyte-reconstituted severe combined immunodeficient (hu-PBL-SCID) mice were infected with pre- or posttreatment isolates and plasma HIV-1 RNA levels and CD4+ T cells were measured. Two of five post-treatment isolates exhibited decreased replication in hu-PBL-SCID mice compared with the paired pretreatment isolate, and both had the V82A mutation in protease associated with resistance to PI. One additional posttreatment isolate with the M184V mutation in reverse transcriptase showed diminished replication. CD4+ T-cell depletion was similar following infection with either the pre- or posttreatment isolates. Subtle losses in the replication capacity of PI- or NRTI-resistant viruses may contribute to relative preservation of CD4+ T-cell counts in persons who experience virologic failure. Cytopathic effects of viral infection for target T cells vary from patient to patient but appear not to be influenced by mutations associated with failure of therapy in this system. Key Words: Viral fitness—Treatment failure—Preservation of CD4+ T cells.
human cells permits sustained virus replication that can be readily measured by plasma HIV RNA assays and infection leads to depletion of CD4+ T cells (9,10). Primary immune responses to HIV infection do not develop in this model, so the kinetics of virus replication reflect viral fitness and the available number of target cells, a function of the rate of CD4+ T-cell depletion (9).

METHODS

Patients

To be eligible to participate in AIDS Clinical Trials Group Protocol 315 (ACTG 315), patients needed to have a baseline CD4+ T-cell count of between 100 and 300 cells/μl, to be zidovudine experienced, but naive to both lamivudine and HIV-1 PI. Patients were given open label zidovudine, lamivudine, and ritonavir, as described in detail elsewhere (11). Five patients participating in this trial were chosen because they had experienced virologic rebound after having experienced a greater than 1 log decrease in plasma HIV-1 RNA copies/ml yet maintained a sustained increase in CD4+ T cell counts (>100 cells/μl > baseline values). Treatment failure did not appear to be related to poor compliance because PI plasma levels were within the expected range.

Virus Isolates

Peripheral blood mononuclear cells (PBMCs) were cultured for HIV-1 isolation according to standard consensus protocols (12). Initial virus isolation was performed before any changes in antiretroviral therapy, and posttreatment isolation was made at the time indicated in Figure 1. Viruses were typed for coreceptor usage by infection of CCR5- or CXCR4-transfected GHOST cells as described (9).

Culture Conditions

Virus replication was assayed in human PBMCs activated by exposure to 5 μg/ml phytohemagglutinin (PHA) for 2 days followed by culture in 25 units/ml of human interleukin (IL)-2 for an additional 3 days. Cultures were maintained in 2 ml of RPMI-1640 culture medium with 10% fetal calf serum in 24-well plates, and 1-ml medium removed for assay of p24 CA antigen by commercial enzyme-linked immunosorbent assay (ELISA) (NEN Life Sciences, Boston, MA, U.S.A.) at the indicated time.

Reconstitution of SCID Mice With Human Peripheral Blood Lymphocytes

C.B-17 SCID mice were engrafted with PBMCs prepared from individual volunteer donors of known CCR5 genotype provided by the General Clinical Research Center at Scripps Clinic as previously described (9). These mice were infected by injection of 5 ng p24 for each virus isolate, so that any differences in infectious titer contributed by PI-induced mutations would be eliminated. The infectious titer/ng p24 varied less than twofold between isolates from one patient, but varied as much as 20-fold between patients. Each virus isolate was coded so that the experiments were double-blind until all data were collected.

Analysis of Plasma Viral Load

Infection of hu-PBL-SCID mice with HIV-1 was determined by plasma HIV-1 RNA levels measured by the quantitative Roche polymerase chain reaction (PCR) assay (Amplicor HIV Monitor, Roche Molecular Systems, Somerville, NJ, U.S.A.). The limit of detection was 200 to 400 copies/ml depending on the plasma volume available.

Measurement of CD4+ T-Cell Depletion

Depletion of CD4+ T cells was measured by flow cytometry. Cells recovered from the peritoneal cavity of hu-PBL-SCID mice were stained with fluorescein- or phycoerythrin-labeled antibodies to human CD3, CD4, CD8, or CD45 and mouse H-2Kd (Pharmingen, San Diego, CA, U.S.A.) and analyzed on a FACSCalibur (Becton Dickinson Immunocytometry Systems, Mountain View, CA, U.S.A.) flow cytometer. CD4+ T-cell counts are expressed as a percentage of total human CD3+ cells recovered.

Sequence Analysis

Nucleotide sequence data were obtained from proviral DNA of cultured HIV-1–infected PBMCs obtained at study entry or after treatment failure. Hybridization-based nucleic acid sequencing was performed using high-density nucleotide arrays as previously described (13). The resulting nucleotide sequences were determined using the “rules” algorithm (GeneChip software, version 2.0, Affymetrix, Santa Clara, CA, U.S.A.). This method is biased toward detection of the predominant sequence in the viral population. Resistance mutations were identified with the use of an online database (14). Before and after treatment sequences have been deposited in Genbank. The accession numbers for before treatment sequences are: patient identifier (PID) 250736 (patient A), AF198398; PID 271764 (patient B), AF198405; PID 271897 (patient C), AF198411; PID 610282 (patient D), AF198415; and PID 610471 (patient E), AF198426. Accession numbers for after treatment isolates are: PID 250736, AF205950; PID 271897, AF205947; PID 610282, AF205948; PID 610471, AF205949; and PID 271764, AF20946. The protease gene of virus recovered from hu-PBL-SCID mice after 4 weeks’ infection was sequenced to rule out compensatory mutations that might change viral fitness. The protease sequence was amplified from proviral DNA by PCR using the 5’ primer GCC-GATAGACAAGGAACTGT and 3’ primer CAGTAAAATTAAAGC and analyzed on a FACSCalibur (Becton Dickinson Immunocytometry Systems, Mountain View, CA, U.S.A.) flow cytometer. CD4+ T-cell counts are expressed as a percentage of total human CD3+ cells recovered.

RESULTS

Comparison of Pre- and Posttreatment HIV-1 Isolates in hu-PBL-SCID Mice

Ten virus isolates from 5 patients participating in ACTG 315 were analyzed. The viral loads and CD4+ T cell counts of these 5 patients are presented in Figure 1. Posttreatment isolates were obtained 12 to 42 weeks after virologic failure, defined by increased plasma viral RNA levels. All 5 patients maintained increased CD4+ T lymphocytes despite virologic failure. Of the 5 patients, 2 (patients B and E) had incurred V82A mutations in protease associated with resistance to ritonavir (14) during
treatment (Table 1). The viral isolate from PID 610471 (patient E) had, in addition, incurred the M184V mutation in RT associated with resistance to lamivudine during the treatment interval, and this same mutation appeared in the posttreatment isolate from PID 250736 (patient A) in the absence of any significant mutations in protease (Table 1). Four of five pretreatment isolates had the K70R mutation associated with resistance to zidovudine (15), and this mutation was retained in the posttreatment isolates (Table 1).

The matched pre- and posttreatment isolates were used to infect groups of five hu-PBL-SCID mice in three suc-
cessive experiments. The mean HIV RNA levels ± standard error are plotted in Figure 2. In a separate in vitro experiment, each virus isolate was used to infect activated PBMCs from a single donor. The mean p24 levels of triplicate cultures are also plotted in Figure 2. Results show that 3 of 5 posttreatment isolates had diminished replication in hu-PBL-SCID mice compared with the matched pretreatment isolate [PID 250736 (patient A), 271764 (patient B), and 610471 (patient E)]. In addition, 2 of 5 posttreatment isolates displayed similar or higher replication patterns compared with the matched pretreatment isolate (PID 271897 [patient C] and 610282 [patient D]). We calculated the cumulative virus burden (area under the curve; AUC) for the paired isolates (Table 2). Posttreatment isolates from patients A, B, and E showed a decline in cumulative virus burden of between 52.6% and 74.7% compared with the pretreatment isolates. These declines in AUC were of marginal significance individually because of increasing variation with duration of infection ($p$ = 0.06–0.10; Student $t$-test). Viral RNA levels at 1 (patient A, patient D) and 2 (patient A) weeks after infection were significantly lower ($p < 0.01$, two-tailed $t$-test) comparing posttreatment with pretreatment isolates. The three posttreatment isolates with trends toward diminished replication were those that incurred either V82A mutations in protease, M184V mutations in RT, or both. Posttreatment isolates without new resistance-associated mutations did not show any suggestion of diminished replication.

The number of residual CD4$^+$ T cells in each animal was also determined after 4 weeks of infection (Fig. 2). Recovery of CD4$^+$ T cells in animals infected with posttreatment isolates was higher than in animals infected with pretreatment isolates in only two pairs of patient isolates (Fig. 2, PID 250736 [patient A] and 610282 [patient D]), and these differences were not statistically significant. One pair of isolates (PID 271897; patient C) consisted of a mixture of R5 and R5X4 viruses. This phenotype correlated with a more rapid depletion of CD4$^+$ T cells and a shorter duration of infection than was seen with the remaining isolates, which all typed as R5 viruses (data not shown). Although the course of viremia was shorter in hu-PBL-SCID mice infected with R5X4 isolates, we did not see evidence of more rapid depletion of CD4$^+$ T cells than was observed previously (9).

Virus was isolated from mice after 4 weeks' infection and the protease gene sequenced to determine whether resistance-conferring mutations had reverted in the absence of drug selection. Although there were 1 to 3 new mutations at known polymorphic sites in protease, no reversion of PI-resistance mutations was observed and no new PI-resistance conferring mutations were seen (data not shown).

**Comparison of Pre- and Posttreatment HIV-1 Isolates in Vitro**

The ability of each pair of isolates to replicate under standard in vitro conditions is also shown in Figure 2. Only two of the posttreatment isolates (PID 610282 [patient C] and 610471 [patient D]) showed any suggestion of diminished replication, and peak p24 levels differed

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<th>Isolate</th>
<th>Protease</th>
<th>RT</th>
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Sequence changes are from consensus clade B sequences. Mutations associated with resistance are in bold.

**Table 1. Sequence changes in protease and reverse transcriptase (RT) before and after treatment regimen containing protease inhibitors**

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FIG. 2. Infection of human peripheral blood leukocyte-reconstituted severe combined immunodeficient (hu-PBL-SCID) (left panels) or peripheral blood mononuclear cell (PBMC) cultures (right panels) with pre- or posttreatment isolates from the 5 patients shown in Figure 1. Each experiment involved 20 to 25 mice reconstituted with PBMCs from a single donor, and each virus isolate was used to infect five individual mice. Plasma viral RNA levels were determined by quantitative polymerase chain reaction at weekly intervals after infection. Left panels: Geometric mean plasma viral RNA levels (in copies/ml) of hu-PBL-SCID mice infected with pretreatment isolates (■) or posttreatment isolates (□). Error bars are ± standard error of the mean. Right panels: In vitro replication of the same isolates measured as the mean pg/ml p24 capsid antigen recovered from triplicate cultures on days 5 to 17 of culture. Error bars are not shown if they are < 5% of the mean. Inset boxes: Mean percentage of CD4+ T cells ± standard error recovered from the peritoneal cavity of HIV-infected hu-PBL-SCID mice infected with pre- or posttreatment isolates after 4 weeks of infection. Uninfected hu-PBL-SCID mice have 30% to 50% CD4+ T cells, so all isolates except those from PID 610282 (D) led to substantial depletion.
< twofold between pre- and posttreatment isolates (differences in AUC were not significant). Although the M184V mutation in RT has been reported to cause diminished replication in vitro (17), posttreatment isolate from PID 250736 (patient A) with the M184V change did not show any difference in replication in these experiments.

**DISCUSSION**

Three results were obtained in this comparison of pre- and posttreatment HIV-1 isolates from patients failed by a PI-containing regimen yet maintaining improved CD4+ T-cell counts. First, two posttreatment isolates with V82A mutations in protease conferring drug-resistance showed diminished replication in hu-PBL-SCID mice. One posttreatment isolate with a M184V mutation in RT also showed diminished replication. Second, there was no clear difference in the ability of any pre- versus posttreatment isolates to cause depletion of CD4+ T cells in infected mice. Third, no substantial difference in replication kinetics was observed in PBMC culture between any pairs of isolates, in accord with some (6) but not all (17) previous observations.

These experiments thus suggest that diminished replication capacity of posttreatment isolates may partially explain the preservation of CD4+ T-cell increases in the presence of virologic failure (3). Mutations known to confer resistance to lamivudine or ritonavir were associated with fivefold to tenfold lower plasma HIV RNA levels when steady state replication was achieved (weeks 2–4 of infection, Fig. 2), and delayed the early increase in viremia in two posttreatment isolates (weeks 1–2 of infection, Fig. 2). A clear difference in the kinetics of plasma viremia and CD4+ T-cell depletion among CCR5-, CCR5/CXCR4-, and CXCR4-using viruses in the hu-PBL-SCID model has previously been demonstrated (9). This finding was confirmed by the two different patterns of viral RNA kinetics observed in these experiments, one corresponding to R5 isolates (PID 250736, 610471, 271764, and 610282) and a second to R5X4 or X4-tropism (PID 271897; patient C).

This apparent decrease in relative virus fitness associated with PI- or nucleoside reverse transcriptase inhibitor (NRTI)–resistance conferring mutations was more obvious in the hu-PBL-SCID mouse model than in PBMC cultures. This result might be caused by more rapid virus RNA turnover in vivo compared with p24 antigen accumulation in tissue culture. Single cycle infection in vitro may be more sensitive in detecting minor deficits in viral replication associated with resistance-conferring mutations (18). In addition, activated CD4+ lymphocytes expressing CCR5 comprise only a minor population of cells in mice, but they are a major population in cell culture. Target cell limitations may accentuate minor differences in the kinetics of virus replication and spread. Protease mutations that decrease the rate of proteolytic processing of viral polyproteins delay the maturation of newly formed virions to full infectivity (19). This delayed virion maturation could be much more important in vivo than in vitro. The M184V mutation in RT impairs a number of functions (20), including attenuated viral growth in vitro in one report (17).

We would thus propose that the suggestion of decreased fitness of posttreatment isolates observed in the hu-PBL-SCID model reflects some of these in vivo constraints on virus spread. One possible explanation for the preservation of CD4+ cells in patients failed by PI-containing regimens may be a reduction in replicative fitness associated with NRTI- or PI-resistance conferring mutations, as suggested by the results with three of five post-treatment isolates studied here. As the CD4+ T cell
depletion rate is related to the magnitude of HIV replication (21), a diminished replicative fitness may be sufficient to explain the relative preservation of immune cells observed. Alternatively, a diminished cytopathogenicity of these isolates that could not be detected in these experiments may also play a role, although these data do not support this hypothesis. A recent study of patients failing similar PI-containing regimens (3) suggests that even temporary suppression of viral load plus residual (if reduced) antiviral activity may lead to prolonged benefits in CD4+ T-cell counts. Further studies are necessary to provide a more complete explanation for the several factors that may contribute to disparity between virologic failure and sustained CD4+ T-cell improvement.

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