thy/liv−SCID-hu Mice: A System for Investigating the In Vivo Effects of Multidrug Therapy on Plasma Viremia and Human Immunodeficiency Virus Replication in Lymphoid Tissues

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Modified, human immunodeficiency virus (HIV)−inoculated thy/liv−SCID-hu mice were used to evaluate the in vivo efficacy of antiretroviral drugs. Ritonavir treatment alone initially suppressed plasma viremia, but the viremia recurred with the appearance of ritonavir-resistant HIV isolates. Multidrug therapy suppressed plasma HIV RNA to undetectable levels; however, plasma viremia returned after therapy was stopped, showing that the therapy did not completely suppress HIV infection in the thymic implant. When thy/liv−SCID-hu mice were treated with a combination of zidovudine, lamivudine, and ritonavir immediately after inoculation with HIV, cocultures of the thymic implants remained negative for HIV even 1 month after therapy was discontinued, suggesting that acute treatment can prevent the establishment of HIV infection. Thus, these modified thy/liv−SCID-hu mice should prove to be a useful system for evaluating the effectiveness of different antiretroviral therapies on acute and chronic HIV infection.

Recent advances in understanding the pathophysiology of human immunodeficiency virus (HIV) infection coupled with the development of potent therapeutic agents have revolutionized the treatment of HIV-infected persons [1]. Treatment with HIV protease inhibitors, such as saquinavir and ritonavir, can markedly decrease HIV virus load and increase peripheral CD4+ T cell counts in HIV-infected persons [2, 3]. These effects can be enhanced significantly by the addition of two other anti-HIV agents, zidovudine and zalcitabine, to the therapeutic regimen [4, 5]. Combination therapy with protease inhibitors and reverse transcriptase (RT) inhibitors, such as zidovudine and lamivudine (3TC), can reduce plasma HIV RNA to undetectable levels and may even prevent HIV infection when given acutely [5, 6].

Because the lymphoid tissues are the major site of HIV replication [7, 8], it is important to evaluate whether these therapeutic approaches also suppress HIV replication in the lymphoid compartment. If they do, it will be necessary to ascertain whether there is still a long-lived, latently infected cell population, such as macrophages, resting T cells, or stem/progenitor cells, that may function as a viral reservoir to be reactivated after the withdrawal of therapy. However, performance of these studies in HIV-infected subjects is limited by the difficulty of frequently accessing lymphoid tissue for analysis. Furthermore, testing the efficacy of newer, unproven antiretroviral drugs and novel drug combinations in patients is restricted by ethical constraints due to the availability of other treatments with known effectiveness. Therefore, there is a pressing need to develop in vivo systems for evaluating the activity of various antiretroviral drug combinations against a spectrum of HIV isolates with different biologic behaviors. There is also a need for evaluating the effect of these treatments on HIV replication in lymphoid tissue reservoirs and the generation and selective outgrowth of drug-resistant isolates.

SCID/SCID C.B-17 mice (SCID mice) implanted with human fetal thymus and liver are an attractive small animal system for studying in vivo HIV-1 infection [9, 10]. By modifying the technique of implanting human fetal thymus and liver into SCID (thy/liv−SCID-hu) mice, we generated thy/liv−SCID-hu mice that had markedly increased numbers of human T cells in the peripheral lymphoid compartment and that developed disseminated HIV infection after intraintplant or intraperitoneal inoculation [11]. These modified thy/liv−SCID-hu mice are also populated with human monocytes, develop plasma viremia, can be infected with a broad range of T cell−tropic and macrophage-tropic HIV isolates, and have been used to demonstrate that
treatment with interleukin-10 can inhibit HIV infection [12, 13]. In the current study, we demonstrate the usefulness of these thy/liv–SCID-hu mice for evaluating the effects of multidrug therapy on in vivo HIV infection.

Materials and Methods

Implantation of human thymic and liver tissue into SCID mice. Fetal thymic and liver tissue (hu-thy/liv) were obtained from 17–to 21–gestational week-old human fetuses within 8 h after the elective termination of pregnancy and implanted into SCID mice (6–8 weeks old) as described [11, 12]. In brief, human fetal thymus and liver tissue obtained from the same fetus were cut into 1-mm³ sections. After the SCID mice were anesthetized with pentobarbital (40–80 mg/kg), the left and right kidneys were sequentially exteriorized and subcapsularly implanted with at least 10 pieces of syngeneic thymus and liver tissue. The procedure was associated with minimal morbidity and mortality, and >95% of the mice were successfully implanted. By 3 months after transplantation, the implanted tissue had increased >20-fold in size.

Flow cytometry. Mononuclear cells were harvested from the hu-thy/liv implants of the thy/liv–SCID-hu mice and stained with peridinin-chlorophyll protein–conjugated monoclonal antibody (MAb) to human CD45, fluorescein isothiocyanate–conjugated MAb to human CD4, and phycoerythrin-conjugated MAb to human CD8 (Becton Dickinson, Mountain View, CA) as described [11]. Expression of human CD4 and CD8 by lymphocytes present in the mice was assessed by three-color flow cytometry, using a cell analyzer and computer software (FACScan and LYSIS-II; Becton Dickinson). Expression of human CD45 was used to confirm the human origin of the cells. Lymphocyte gates were set on the basis of forward- and side-scatter profiles corresponding to gates set for control human lymphocytes from healthy adult volunteers. Nonviable cells and unlysed red blood cells were excluded from analysis on the basis of their forward- and side-scatter profiles. Cutoff values for the quadrants as well as compensation for phycoerythrin versus fluorescein isothiocyanate versus peridinin-chlorophyll protein emission was determined on the basis of single, double, and triple staining of positive (human adult) and negative control (C.B-17 mouse) mononuclear cells with antibodies to human CD4, CD8, and CD45 and staining with the appropriate mouse IgG isotype controls.

Infection of thy/liv–SCID-hu mice with HIV. The HIV strain used in this study, HIV-1$_{10}$, is a primary, monocytie-tropic, nonsyncytium-inducing isolate that was derived from peripheral blood mononuclear cells (PBMC) from a 17-month-old HIV-1–infected child. The strain, which was derived by coculture with phytohemagglutinin (PHA)-activated donor PBMC, was expanded by another round of coculture with PHA-activated PBMC and then divided into aliquots that were frozen in liquid nitrogen as described [12]. Mice were infected by direct injection of 300 TCID$_{50}$ of HIV-1$_{10}$ in a volume of 30 µL into the left hu-thy/liv implant. To control for variability due to the source of donor tissue, mice engrafted with the same donor tissue were distributed into each treatment group. All tissue was screened and shown to be homozygous for the normal CCR5 allele.

Administration of drugs and measurement of saquinavir and ritonavir levels. Drugs were administered to the mice by mixing the indicated drugs with powdered animal feed and then adding the mixture to feeding jars that were designed to minimize spillage. The drug dosage for each mouse (each weighing ~25 g) were calculated on the basis of their average oral intake of 5 g of feed/day. The thy/liv–SCID-hu mice were housed singly so that drug consumption could be confirmed by measurement of the quantity of feed consumed. Saquinavir and ritonavir in plasma samples were quantitatively analyzed by Oneida Research Services (Whitesboro, NY), using a specific high-pressure liquid chromatography (HPLC)–UV method. Unknown, spiked standard and quality control samples were extracted from plasma with an organic solution and then evaporated to dryness with nitrogen, reconstituted in an aqueous solution, and analyzed by reverse-phase HPLC within a linear range of 100–3500 ng/mL. Standard curves were constructed from the peak height ratios of the drug to internal standards, using weighted linear regression analysis. Plasma levels of quality control samples and ‘‘unknowns’’ were then calculated with respect to the standard curve.

Titration of HIV-infected mononuclear cells in the hu-thy/liv implant by limiting-dilution coculture. The number of HIV-1–infected mononuclear cells present in the hu-thy/liv implants was measured by quantitative coculture as described [12]. Five-fold dilutions (ranging from 1 × 10⁶ to 3.2 × 10⁵ cells) of mononuclear cells from the hu-thy/liv implants were cultured in quadruplicate at 37°C in 24-well plates with PHA-activated donor mononuclear cells (1.0 × 10⁵) in 2.0 mL of RPMI 1640 containing fetal calf serum (10% vol/vol) and interleukin-2 (32 U/mL). After 1 to 2 weeks, the p24 antigen content of the culture supernatant was measured using the HIV-1 p24 core profile ELISA assay (DuPont NEN, Wilmington, DE). The lowest number of added mononuclear cells that infected at least half of the quadruplicate cultures with HIV-1 was taken as the end point or TCID; data are presented as TCID/10⁶ mononuclear cells as described [12].

Quantitation of plasma HIV RNA levels. Amplicor HIV-1 monitor kits (provided by Roche Diagnostic Systems, Branchburg, NJ) were used, according to the manufacturer’s recommended protocol, to quantitate HIV RNA in mouse plasma. The kits can quantitate virion-associated HIV-1 RNA in plasma at concentrations as low as 400 RNA copies/mL. The internal HIV quantitation standard (QS), a synthetic RNA molecule with primer binding sites identical to the HIV target and a unique internal probe sequence specific to the QS RNA molecule, was added to 200 µL of each plasma sample. RNA was then extracted with a lysis reagent containing guanidine thiocyanate, precipitated with isopropanol, and resuspended in reaction buffer. A 142-bp sequence in the HIV gag gene was amplified by reverse transcription and polymerase chain reaction (PCR), using recombinant Thermus thermophilus DNA polymerase and biotinylated primers SK431 and SK462. PCR product contamination was controlled by incorporating dUTP during the PCR reaction and then treating all samples with AmpErase (from the Amplicor kit) prior to PCR to eliminate carryover of any dUTP-containing PCR product.

The biotinylated HIV and QS amplicons were detected in separate wells of a microwell plate coated with HIV-specific and QS-specific oligonucleotide probes, respectively, using avidin–
horseradish peroxidase conjugate and a colorimetric reaction for horseradish peroxidase. To measure the HIV and QS amplicons in a dynamic range from 400 to 750,000 copies/mL, 5-fold serial dilutions of the amplicons were made in the HIV-specific and QS-specific wells of the microplate (rows A–F and G–H, respectively). The HIV RNA copy number was then calculated from the known input copy number of the QS RNA, the optical densities at 450 nm of the HIV and the QS wells that fell within a defined range, and the dilution factors associated with the selected wells.

Sequence analysis of the protease gene. DNA was isolated from the HIV-infected hu-thy/liv implants, and the protease coding region was amplified by PCR, using the primers and conditions described [14]. The amplified products were cloned (TA cloning kit; InVitrogen, Carlsbad, CA) and sequenced using the dideoxy-chain termination method.

Statistical analysis. The Student’s t test for unpaired data was used for all statistical comparisons.

Results

Pharmacokinetic analysis of absorption of saquinavir and ritonavir. Saquinavir and ritonavir are symmetry-based HIV protease inhibitors that inhibit proteolysis of the gag and gag-pol proteins and render newly formed HIV particles noninfectious [2, 3]. To determine whether therapeutic levels of these drugs could be obtained in mice by oral administration, we mixed saquinavir alone (2500 mg/kg), ritonavir alone (200 mg/kg), or a combination of saquinavir-ritonavir (2500 and 200 mg/kg, respectively) with powdered animal feed and administered it to the mice in spill-resistant feeding jars. The saquinavir-ritonavir combination was examined because ritonavir dramatically increases saquinavir levels in animals and humans [15] by inhibiting CYP3A4, the isoform of cytochrome P450 that is responsible for the metabolic clearance of saquinavir, thereby potentially enhancing the anti-HIV activity of the drugs [16].

After mice were treated for 2 weeks, 3 blood samples were obtained 6 h apart, and the plasma levels of the drugs were determined (figure 1). Mice treated with ritonavir alone (n = 4) had levels that ranged from 6000 ng/mL in the late morning to 2000 ng/mL at night and that were comparable to peak levels reported for humans treated with one 500-mg dose of ritonavir [17]. Saquinavir-treated mice (n = 4) had plasma levels of 200–600 ng/mL, which were similar to levels in humans treated with 7200 mg of saquinavir/day [18].

Plasma levels of ritonavir in mice treated with the ritonavir-saquinavir combination did not differ significantly from those in mice treated with ritonavir alone (P < .31). In contrast, plasma levels of saquinavir in mice treated with the drug combination were significantly increased compared with levels in mice treated with saquinavir alone (P < .02).

Taken together, these data indicated that therapeutic levels of ritonavir and saquinavir were obtained in mice treated by oral administration of drug admixed with their feed and that saquinavir levels could be markedly enhanced by the coadministration of ritonavir.

Evaluation of the kinetics of HIV replication in thy/liv–SCID-hu mice. Because therapeutic levels of ritonavir were obtained in mice after oral administration of the drug and because our HIV-infected thy/liv–SCID-hu mice developed plasma viremia [13], the kinetics of HIV replication in these mice were determined by measuring the rate of plasma HIV RNA decline after the initiation of ritonavir therapy as described in studies with HIV-infected persons [19]. Six mice were infected by intraintestinal injection with HIV-1<sub>39</sub>, and 5 weeks later, after steady-state plasma HIV RNA levels were attained, the mice were started on ritonavir treatment (200
The temporal response of plasma HIV RNA levels to ritonavir therapy in a representative HIV-infected thy/liv–SCID-hu mouse example is shown in figure 2. Attainment of a steady state virus load before the initiation of therapy was indicated by the measurement of similar levels of HIV RNA in the plasma at two time points that were 7 days apart. No significant decrease in HIV RNA levels was observed during the first 3 days of treatment, but between the fourth and eighth days of therapy, plasma HIV RNA levels declined rapidly to <5% of the baseline levels. These low levels of plasma viremia persisted for an additional 7 to 8 days, after which time, HIV RNA was no longer detectable in the plasma.

Analysis of the acute drop in HIV RNA levels with the mathematical model described by Perelson et al. [19] for in vivo HIV dynamics in HIV-infected humans was used to determine the decay rate constants for plasma virions (c) and infected cells (δ) and the total virion production in the HIV-infected thy/liv–SCID-hu mice (table 1). In mice analyzed with this model, the mean average life span of an extracellular virion was 0.34 days and of a productively infected cell was 1.89 days.

In vivo development of ritonavir-resistant isolates during prolonged monotherapy. After the 6 thy/liv–SCID-hu mice were treated with ritonavir for 16 days, they had no detectable HIV RNA in their plasma. When ritonavir treatment was stopped in 4 of these mice, plasma viremia recurred within 2 days and returned to pretreatment levels 6 days later. Of great interest was the observation that despite being continued on ritonavir, the other 2 thy/liv–SCID-hu mice, 216c4 and 216c2, had recurrence of plasma viremia 37 days and 3 months after treatment was started, respectively. The degree of cellular HIV infection in these 2 mice was evaluated by determining the number of HIV-infected cells in a hu-thy/liv implant biopsy: The implants had significant HIV infection (3125 and 25 TCID/10⁶ thymocytes, respectively) despite the continued ritonavir treatment.

To determine if the HIV-1 isolates infecting these mice had developed a decreased sensitivity to ritonavir, the initial inoculum of HIV-1₅₉ and the HIV isolated from these hu-thy/liv implants after 3 months of ritonavir treatment were tested for their sensitivity to ritonavir as described [20]. In contrast to the initial inoculum of HIV-1₅₉, which had an IC₉₀ of ritonavir of ~0.1 μM, HIV isolated from the ritonavir-treated thy/liv–SCID-hu mice had an ~3-fold (216c4 isolate) or 7-fold (216c2 isolate) higher IC₉₀ of ~0.3 μM and 0.7 μM, respectively (figure 3). The greater resistance to ritonavir that was exhibited by the HIV isolated from mouse 216c2 (compared with the HIV from mouse 216c4) was paralleled by a higher virus load in the 216c2 hu–thy/liv implant.

To determine if the appearance of isolates displaying phenotypic resistance to ritonavir was associated with specific genotypic changes in the protease gene, proviral DNA from the biopsied hu-thy/liv implants was amplified by PCR, using primers that flanked the protease gene, and then sequenced. In contrast to the protease gene sequence in the initial inoculum of HIV-1₅₉, which had an IC₉₀ by ritonavir of ~0.1 μM, HIV isolated from the ritonavir-treated thy/liv–SCID-hu mice had an ~3-fold (216c4 isolate) or 7-fold (216c2 isolate) higher IC₉₀ of ~0.3 μM and 0.7 μM, respectively (figure 3).

Comparison of the effects of multidrug therapy on plasma viremia and HIV infection in the hu-thy/liv implant. Suppression of cellular HIV infection and plasma viremia by multidrug therapy in our modified thy/liv–SCID-hu mice was investigated by treatment either with a combination of RT and protease inhibitors (zidovudine-3TC-ritonavir) that markedly suppress plasma viremia in HIV-infected persons [5] or with two protease inhibitors (saquinavir and ritonavir). The mice were infected with HIV-1₅₉ by direct inoculation of 300 TCID₅₀ into the left hu-thy/liv implant, and the mice were evaluated 1 month later (by which time systemic HIV infection occurs [13]) by measuring plasma HIV RNA levels and performing quantitative coculture on a biopsy of the left hu-thy/liv implant. Some mice were then started on a combination of zidovudine-3TC-ritonavir (100, 100, and 200 mg/kg/day, respectively; n = 3) or zidovudine-3TC-ritonavir (10, 10, and 20 mg/kg/day, respectively; n = 3); others were started on ritonavir-saquinavir (200 and 2500 mg/kg/day, respectively; n = 2).

After 1 month of therapy, the effect of these therapeutic regimens on HIV infection was determined by measurement...
Table 1. Rate of HIV-1 clearance, infected cell loss and virion production.

<table>
<thead>
<tr>
<th>Mouse</th>
<th>No. of baseline plasma virions/mL</th>
<th>Virion clearance</th>
<th>Infected cell loss</th>
<th>Total virion production (10^4/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse no.</td>
<td>c (day^(-1))</td>
<td>t_1/2 (days)</td>
<td>c (day^(-1))</td>
<td>t_1/2 (days)</td>
</tr>
<tr>
<td>216a2</td>
<td>2259</td>
<td>2.86</td>
<td>0.24</td>
<td>0.49</td>
</tr>
<tr>
<td>216a5</td>
<td>4484</td>
<td>1.46</td>
<td>0.48</td>
<td>0.49</td>
</tr>
<tr>
<td>216c1</td>
<td>4211</td>
<td>4.72</td>
<td>0.15</td>
<td>0.37</td>
</tr>
<tr>
<td>216c2</td>
<td>12,737</td>
<td>4.65</td>
<td>0.15</td>
<td>0.79</td>
</tr>
<tr>
<td>216c4</td>
<td>2309</td>
<td>1.96</td>
<td>0.36</td>
<td>0.56</td>
</tr>
<tr>
<td>216c5</td>
<td>1711</td>
<td>1.91</td>
<td>0.37</td>
<td>0.50</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>4618 ± 4133</td>
<td>2.93 ± 1.44</td>
<td>0.29 ± 0.13</td>
<td>0.53 ± 0.14</td>
</tr>
</tbody>
</table>

NOTE. Baseline plasma virion (containing 2 RNA copies) values are average of measurements taken 7 days and 1 day before treatment was started. To analyze plasma HIV RNA levels (V) on days 3–8, values for rate constant of virion clearance (c), rate of loss of virus-producing cells (d), and half life (t_1/2) were calculated according to formula of Perelson et al. [19]:

\[ V(t) = V_0 \exp(-ct) + ce^c(c-d) \exp(-ct) \exp(-dt) \exp(-ct) \]. Total virion production was calculated based on assumption that plasma and extracellular fluid were in equilibrium.

Figure 3. Sensitivity of HIV from ritonavir-treated thy/liv SCID-hu mice to ritonavir. HIV was isolated by coculture from hu-thy/liv implants obtained 3 months after mice with established HIV infection were started on ritonavir (200 mg/kg/day). Aliquot of coculture supernatant was harvested and added to new cultures containing activated peripheral blood mononuclear cells (PBMC) and indicated concentration of ritonavir. In parallel, aliquot of initial inoculum, HIV-1_b, was also cultured with activated PBMC and indicated concentration of ritonavir. After 7 days, culture supernatants were harvested, and mean p24 concentration (pg/mL) of duplicate culture supernatants was determined to be ~60,000 pg/mL for each isolate. Data are presented as % inhibition of level of infection in untreated culture by indicated concentration of ritonavir.

To determine whether these few infected cells could function as an infectious reservoir, we took some mice off therapy. One month later, the plasma HIV RNA levels and virus loads in a repeat biopsy of the hu-thy/liv implant were measured. Plasma viremia recurred in all of the multidrug-treated thy/liv-SCID-hu mice examined, including 1 mouse treated with high-dose zidovudine-3TC-ritonavir (2832 copies of HIV RNA/mL), 2 mice treated with lower-dose zidovudine-3TC-ritonavir (7410 and 947 copies of HIV RNA/mL), and 1 mouse treated with ritonavir-saquinavir (1004 copies of HIV RNA/mL). This was paralleled by a marked increase in the virus loads in the hu-thy/liv implants of the mouse treated with high-dose zidovudine-3TC-ritonavir (625 TCID/10^6 cells), the 2 mice treated with lower-dose zidovudine-3TC-ritonavir (both 3125 TCID/10^6 cells), and the mouse treated with ritonavir-saquinavir (3125 TCID/10^6 cells).

In the human thymic implants of the thy/liv–SCID-hu mice treated with zidovudine-3TC-ritonavir, a dose-responsive decrease in the CD4/CD8 double-positive thymocyte population occurred that was not observed in untreated mice or mice treated with ritonavir-saquinavir (figure 4C). Because thymopoiesis in the human thymic implant depends on continued hematopoietic activity by the coimplanted human fetal liver to provide new pre-T cells [9], production of immature thymocytes may be inhibited by the suppressive effects of zidovudine and 3TC on hematopoiesis [22, 23]. Therefore it is likely that zidovudine- and 3TC-mediated inhibition of hematopoietic activity in the implanted human fetal liver decreased the production of human precursor T cells and was thereby causing the depletion of the immature CD4/CD8 double-positive population in the implanted human fetal thymus.

Prevention of acute HIV infection by multidrug therapy. To determine if multidrug therapy could prevent HIV infection, we inoculated HIV into the left thy/liv implant of thy/liv–SCID-hu mice. Some of the mice were left untreated (n = 12), while others were immediately started on therapy with
Figure 4. Effect of multidrug therapy on HIV infection in thy/liv-SCID-hu mice. Left thy/liv implants of mice were injected with 300 TCID_{50} of HIV-1, and 1 month later, implants were biopsied and analyzed. Groups of mice were not treated or treated with various combinations and doses of drugs. After 1 month, left thy/liv implant was re-biopsied and reanalyzed. A, Mean no. (SE) of copies of HIV RNA/mL of mouse plasma for each treatment group 1 month after infection and 2 months after infection after receiving 1 month of indicated treatment. B, Mean (SE) virus load in left hu-thy/liv implants for each treatment group 1 month after infection and 2 months after infection after receiving indicated treatment for 1 month was assayed by quantitative coculture. C, Lymphocytes isolated from hu-thy/liv implants from mice 1 and 2 months after infection were assayed by 3-color flow cytometry for expression of human CD4 and CD8. Data for each treatment group are mean % (SE) of thymocytes in implants single positive or double positive (CD4/CD8) for CD4 and CD8. AZT = zidovudine.

zidovudine-3TC-ritonavir (100, 100, 200 mg/kg/day, respectively; n = 5), zidovudine-3TC-ritonavir (10, 10, 20 mg/kg/day, respectively; n = 4), ritonavir-saquinavir (200 and 2500 mg/kg/day, respectively; n = 4), or ritonavir-saquinavir (20 and 250 mg/kg/day, respectively; n = 5). After 1 month of treatment, the injected hu-thy/liv implants were biopsied and cellular HIV infection was evaluated by quantitative coculture (figure 5A). HIV was detected in the hu-thy/liv implants from all untreated mice and from all 5 mice treated with the lower dose of ritonavir-saquinavir. HIV was not detected in implants from any of the 5 mice treated with the higher dose of zidovudine-3TC-ritonavir (P = .013). Partial protection was observed in mice treated with the other treatment regimens: No HIV was detected in the implants from 3 of 4 mice treated with the
Figure 5. Effect of multidrug therapy on prevention of HIV infection in thy/liv±SCID-hu mice. Left thy/liv implants of mice were injected with 300 TCID$_{50}$ of HIV-1$_{99}$. Groups of mice were not treated or immediately treated with various combinations and doses of drugs. After 1 month, left hu-thy/liv implants were biopsied and analyzed. Drug therapy was stopped, and 1 month later, left and right thy/liv implants from 3 mice from each group (4 from low-dose ritonavir-saquinavir group) were analyzed. A. Mean virus load ($\pm$SE) in hu-thy/liv implants from mice 1 month after infection and 2 months after infection, when mice had been off therapy for 1 month, was determined by quantitative coculture. B, Mean ($\pm$SE) plasma levels of HIV RNA in mice 2 months after infection. C, Lymphocytes isolated from hu-thy/liv implants from mice 1 and 2 months after infection were assayed by 3-color flow cytometry for expression of human CD4 and CD8. Data for each treatment group are mean % ($\pm$SE) of thymocytes in implants single positive or double positive (CD4/CD8) for CD4 and CD8. AZT = zidovudine.

lower dose of zidovudine-3TC-ritonavir ($P = .03$) or in the implants from 3 of 4 mice treated with the higher dose of ritonavir-saquinavir ($P = .033$).

Because prevention, as opposed to suppression, of HIV infection by multidrug therapy would be indicated by the continued absence of infection after therapy was discontinued, drug treatment was stopped for all mice. One month later, the mice were reevaluated for HIV infection by quantitative coculture of the left and right hu-thy/liv implants (figure 5A) and by measurement of plasma HIV RNA (figure 5B). Unfortunately, some of the mice died after the first biopsy and were, therefore, not available for reanalysis.

In the surviving mice, HIV infection was still not detectable by coculture of the thymic implants in the hu-thy/liv implants or the plasma from 3 of 3 mice that had been treated with the
Discussion

In the current study, we report on the utility of our modified thy/liv–SCID-hu mice to study the in vivo effects of antiretroviral drug therapy on HIV infection. After inoculation with HIV, these mice developed extensive HIV infection in the hu-thy/liv implants and plasma viremia that displayed rapid turnover, with an average life span of 0.34 days. The high level of HIV replication in the thy/liv–SCID-hu mice was further indicated by the rapid appearance of HIV with decreased sensitivity to ritonavir during monotherapy with ritonavir.

We demonstrated that these mice could be used as a system to correlate the effects of combination drug therapy on HIV cellular infection in the thy/liv implant with the effect on plasma viremia. Multidrug therapy of thy/liv–SCID-hu mice with established HIV infection with either zidovudine-3TC-ritonavir or saquinavir-ritonavir suppressed plasma viremia to undetectable levels and potently decreased cellular infection in the hu-thy/liv implants, but plasma viremia recurred after therapy was stopped. However, immediate treatment with high doses of zidovudine-3TC-ritonavir was effective in preventing HIV infection, which was indicated by the fact that HIV could not be cultured from the hu-thy/liv implants of these thy/liv–SCID-hu mice even after they were off drug therapy for 1 month.

High levels of HIV RNA are present in the plasma of HIV-infected persons during all stages of HIV infection and may be used as an indicator of the degree of viral replication occurring in the lymphoid tissues [24]. The rapid rate by which plasma HIV RNA levels decline in HIV-infected persons after treatment with potent antiretroviral drugs indicates that the plasma HIV population turns over rapidly, contains the most recently replicated HIV, and may be used as a sensitive indicator of the effects of therapeutic interventions on HIV replication [6, 19, 25, 26].

Using a mathematical model described for HIV-infected persons [19], we analyzed the ritonavir-induced decline in plasma HIV RNA levels in HIV-infected thy/liv–SCID-hu mice. The average life span of an extracellular virion was ~0.34 days, with a \( t_{1/2} \) of 0.29 days, and the average life span of productively infected cells was ~1.89 days, with a \( t_{1/2} \) of 1.37 days. These results were comparable to those reported for HIV-infected persons with an average extracellular virion life span of 0.3 days, with a \( t_{1/2} \) of 0.24 days, and an average life span of productively infected cells of 2.2 days, with a \( t_{1/2} \) of 1.6 days [19]. Because these mice do not mount a detectable human or mouse response to HIV [12], these data suggest that the rate of viral and cellular turnover is predominantly a function of HIV replication and not of the immune response directed against HIV. Nevertheless, because turnover of HIV-infected thymocytes may differ from that of HIV-infected peripheral T cells, the mathematical model for T cell decay proposed for HIV-infected patients may not be applicable for the analysis of the kinetics of infected T cell turnover in our hu-thy/liv–SCID-hu mice.

The mice displayed lower total daily virion production (6.2 \( \times 10^9 \) virions/day) than that reported in humans (10.3 \( \times 10^9 \) virions/day) [19], but this was expected considering the ~3000-fold–larger body mass of humans compared with mice and the smaller population of human T cells present in the mice. Although plasma HIV levels rapidly declined by 95% within 8 days after the initiation of ritonavir, it took an additional 7 to 8 days until HIV RNA was no longer detectable in the plasma. It is likely that the second phase of viral decay observed in these thy/liv–SCID-hu mice is attributable to the release of virus into the plasma by secondary sources, such as infected macrophages or dendritic cells, the activation of latently infected T cells or the release of trapped virions, as recently described for HIV-infected persons [27].

Taken together, these results demonstrate that the kinetics of HIV replication in our thy/liv–SCID-hu mice closely resemble that reported in HIV-infected persons and suggest that these mice should be a useful model to study in vivo HIV infection.

Although plasma viremia was initially completely suppressed in thy/liv–SCID-hu mice by treatment with ritonavir, it recurred despite continuation of ritonavir therapy and was associated with extensive infection of the hu-thy/liv implants. HIV isolated from the implants was 3- to 7-fold less susceptible to ritonavir than the initial HIV inoculum, indicating that HIV replicated in the infected thy/liv–SCID-hu mice at a rate suffi-
cient to generate mutations that could confer resistance to rita-
navir [6]. This was confirmed by demonstrating that genotypic
changes associated with the development of ritonavir resistance
were present in the protease gene of the HIV isolated from
thy/liv–SCID-hu mice, a valine-to-alanine change at position
82 (V82A) in mouse 216c4 and an isoleucine-to-valine substi-
tution at position 84 (I84V) in mouse 216c2 [21]. These two
mutations develop in ritonavir-treated patients, with the V82A
change being the most frequently observed mutation, and in
most patients, the first to emerge [28, 29].

The association between the recurrence of plasma viremia
with the appearance of ritonavir-resistant isolates in the rito-
avir-treated mice was similar to that observed in ritonavir-
treated patients [29]. Thus, these thy/liv–SCID-hu mice should
prove to be a useful in vivo system for investigating the emer-
gence of drug-resistant isolates, including the role of variables,
such as drug dosing, and the use of different drug combinations.

Because we demonstrated that therapeutic levels of antiretrovi-
al drugs could be obtained by oral administration, these modified
thy/liv–SCID-hu mice could be used to investigate the effect of
multidrug therapy, given by dietary admixture, on lymphoid tissue
infection and plasma viremia. Previous studies had demonstrated
that SCID-hu mice could be used to assess the in vivo antiviral
effects of zidovudine after HIV was inoculated into their human
thymic implants [30]. However, the resultant HIV infection in
those SCID-hu mice was limited to the thymic implant and was
not associated with the presence of plasma viremia. Therefore,
the use of our modified thy/liv–SCID-hu mice, which develop
high levels of disseminated HIV infection, to correlate the effect
of multidrug therapy on plasma viremia with its effect on the
level of cellular infection represents a major advance for assessing
the in vivo effect of antiretroviral therapy.

The mice were treated with a combination of two RT inhibi-
tors (zidovudine and 3TC) and a protease inhibitor (ritonavir)
or with a combination of two protease inhibitors (ritonavir and
saquinavir). Ritonavir was used as a representative protease
inhibitor, and comparable effects could presumably have been
achieved by a similar combination of different protease and
RT inhibitors. The combination of ritonavir and saquinavir was
chosen to take advantage of the inhibitory effect of ritonavir
on the metabolic clearance of saquinavir [15].

The presence of plasma viremia in the HIV-infected thy/liv–
SCID-hu mice permitted us to examine the effect of treatment
with multidrug therapy on plasma HIV levels and to correlate
it with the level of infection in the hu-thy/liv implant. Plasma vire-


ma was suppressed to undetectable levels (<400 copies/mL) in
all of the mice after 1 month of treatment with zidovudine-3TC-
ronavir (100, 100, and 200 mg/kg/day or 10, 10, and 20 mg/kg/
day, respectively) or ritonavir-saquinavir (200 and 2500 mg/kg/
day, respectively). The level of infection in the hu-thy/liv implants
was significantly decreased by treatment with these multidrug
regimens, but low levels of HIV infection were still detectable
in the hu-thy/liv implants, a result comparable to that reported for
lymphoid tissues in HIV-infected patients treated with multidrug
therapy [31]. One month after therapy was stopped in these mice,
plasma viremia recurred in tandem with increased cellular infec-
tion in the hu-thy/liv implants.

In addition to investigating the effect of multidrug therapy
on established HIV infection, we also examined the capacity
of multidrug therapy initiated immediately after inoculation
with HIV to prevent subsequent in vivo HIV infection. We
demonstrated that different therapeutic regimens had different
capacities to prevent HIV infection. Although HIV could not
be detected by coculture in the hu-thy/liv implants immediately
after treatment with the lower dosage of zidovudine-3TC-rito-
avir (10, 10, and 20 mg/kg/day, respectively) or with ritonavir-
saquinavir (200 and 2500 mg/kg/day, respectively) was
stopped, active HIV infection was present 1 month after drug
therapy was terminated. These data indicated that HIV infection
had been suppressed by these treatments, not prevented, and,
therefore, after therapy was discontinued, extremely low levels
of HIV present in these lymphoid tissues served as a reservoir
to initiate recurrence of active HIV infection.

In contrast, when thy/liv–SCID-hu mice were treated with
the higher dosage of zidovudine-3TC-ritonavir (100, 100, 200
mg/kg/day, respectively), no HIV was detected in the hu-thy/
liv implants by coculture even 1 month after therapy was
stopped. This indicates that when given at the appropriate dose,
the combination of RT inhibitors with protease inhibitors might
successfully prevent HIV infection.

These observations have major potential implications for the
evaluation and clinical treatment of persons treated with potent
anti-HIV therapy soon after acute infection, such as infants
born to HIV-infected mothers or persons presenting acutely
after high-risk exposure. Although this combination appeared
to be more effective in preventing HIV infection than the com-
bination of two protease inhibitors, ritonavir and saquinavir, it
was associated with significant suppression of thymopoiesis in
the hu-thy/liv implant. It is possible that HIV infection would
be detectable in the hu-thy/liv implants of these mice if they
were examined after being without therapy for >1 month.
However, because the hu-thy/liv implant contains a high concen-
tration of thymocytes that are extremely sensitive to HIV
infection, it is likely that any HIV present in tissue would have
initiated a detectable infection by 1 month, as observed in the
mice treated with lower doses of zidovudine-3TC-ritonavir or
with ritonavir-saquinavir.

Although the thy/liv–SCID-hu model described here repre-
sents a significant advance over in vitro studies, the absence
of HIV-specific human immune responsiveness in these thy/
liv–SCID-hu mice [12] may underestimate the effect of drug
treatment and alter the degree of HIV infection occurring in
the lymphoid tissues. Therefore, results obtained using these
mice should be interpreted with caution pending confirmation
with human studies. Our modified thy/liv–SCID-hu mice
should prove to be a useful system for evaluating whether drug
combinations that include protease inhibitors can eradicate or just suppress HIV infection in lymphoid tissue, the major location for HIV replication.

The availability of an animal model will be especially important for experiments that will be difficult to perform in patients due to ethical constraints, such as those designed to observe whether HIV infection becomes reactivated after the termination of therapy, and for examining the effects of multidrug therapy against drug-resistant HIV isolates. Since thy/liv- and in combination with ritonavir in HIV-infected patients. AIDS 1997;11:F29–33.

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