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Use of Standardized SCID-hu Thy/Liv Mouse Model for Preclinical Efficacy Testing of Anti-Human Immunodeficiency Virus Type 1 Compounds†

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We have developed standardized procedures and practices for infection of SCID-hu Thy/Liv mice with human immunodeficiency virus type 1 for the prophylactic administration of antiviral compounds and for evaluation of the antiviral effect in vivo. Endpoint analyses included quantitation of viral load by intracellular p24 enzyme-linked immunosorbent assay, DNA PCR for the presence of proviral genomes, flow cytometry to measure the representation of $CD4^+$ and $CD8^+$ cells, and cocultivation for the isolation of virus. Efficacy tests in this model are demonstrated with the nucleoside analogs zidovudine and dideoxyinosine and with the nonnucleoside reverse transcriptase inhibitor nevirapine. This small-animal model should be particularly useful in the preclinical prioritization of lead compounds within a common chemical class, in the evaluation of alternative in vivo dosing regimens, and in the determination of appropriate combination therapy in vivo.

The process of drug discovery and development is facilitated by efficient and predictive decision-making trees capable of selecting the best compound among thousands. Unfortunately, since many disease etiologies in humans are organ and/or species specific, it has been difficult to test preclinical leads for efficacy in tissue culture or animal models. Thus, proof of efficacy for a given lead compound usually awaits the completion of lengthy, and hence costly, human clinical trials (8).

The problems associated with this decision-making tree have been very apparent in the search for compounds effective against human immunodeficiency virus type 1 (HIV-1) (18). $CD4^+$ cells permissive for HIV-1 infection can be cultured and used to test the efficacies of putative anti-HIV-1 compounds in vitro. Such tests are, however, not necessarily predictive of efficacy in vivo: the HIV-1 isolates which grow in vitro are dissimilar from those that replicate in vivo; the cell types which proliferate in tissue culture are not representative of those found within HIV-1-infected organ systems in humans; indeed, since most cells in vivo are not proliferating at all, even the cell cycle status of the tissue culture assay is largely irrelevant. Additionally, many lead compounds fail in vivo as a result of basic pharmacologic considerations, e.g., metabolism to an inactive compound, failure to be metabolized to an active compound, excretion or rapid turnover, and poor bioavailability. Perhaps as a consequence, a variety of compounds which demonstrated good efficacy profiles against HIV-1 in vitro (e.g., dextran sulfate or a Tat inhibitor) (9, 30) have failed when they were finally introduced into human clinical trials. Reciprocally, in the case of truly active compounds, it is likely that in vitro efficacy profiles have forced the inappropriate prioritization of structurally related congeners.

With an ever-increasing number of candidate drugs for use as therapies against HIV-1, the importance of a preclinical screening mechanism to complement in vitro assays has increased. The SCID-hu mouse model (19) was developed to serve as a preclinical model for the evaluation of HIV-1 pathogenic mechanisms in vivo and for the prioritization of antiviral compounds which demonstrate in vitro efficacy. This mouse model is constructed by transplantation of interactive human lymphoid organs into the immunodeficient C.B-17 *scid/scid* stock. In previous studies, an infection protocol for mice engrafted with human fetal lymph nodes was established (15). This model was quite effective as a research tool: following intravenous inoculation of 1×10^5 to 2×10^5 50% tissue culture infective doses ($TCID_{50}$s) of low-passage-number viral stocks, infection was detected in 100% of animals at a 2-week endpoint. Continued use of the model, however, made it clear that it was extremely sensitive to environmental conditions in the SCID mouse colony and not robust enough for large-scale preclinical testing.

As an alternative to the lymph node model, we have now developed a SCID-hu model using conjoint implants of human fetal thymus and liver (to create SCID-hu Thy/Liv mice). These implants have been shown previously to be vascularized and to grow when implanted beneath the kidney capsule, eventually reaching a total mass of 10^7 to 10^8 human cells (22). Such growth occurs in 80 to 90% of recipients and is relatively unaffected by variable parameters in the SCID mouse colony. For periods of time as long as 12 months in vivo, the Thy/Liv organs sustain multilineage human hematopoiesis and provide for a continuous source of normal human $CD4^+$ T cells (17, 22, 27, 28). Viral replication can be observed in a time- and dose-dependent manner after inoculation of HIV-1 (21), and in the case of some virus isolates, thymocyte depletion is observed within a 3- to 5-week time interval (2, 3, 14, 25). Such depletion includes loss of $CD4^+$ $CD8^+$ immature cortical thymocytes and an inversion of the CD4/CD8 ratio within the thymic

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medulla and in the peripheral blood. Several mechanisms have been associated with these events, including indirect, apoptotic destruction of uninfected thymocytes and direct infection and destruction of intrathymic $CD3^-$ $CD4^+$ $CD8^-$ progenitor cells (26). Upon administration of antiviral agents such as zidovudine (AZT) to the SCID-hu Thy/Liv mouse, HIV-1 replication is inhibited (20).

We describe here a SCID-hu Thy/Liv model that has been optimized for the preclinical evaluation of antiviral agents. Methods for the production of viral stocks, for infection of SCID-hu mice, for administration of antiviral compounds, and for endpoint analysis of antiviral efficacy are provided. By using the current model, single antiviral agents can be tested in a dose-ranging manner against four standardized virus stocks, including two molecularly cloned viruses and two primary isolates. The effects of such agents on the course of HIV-1 replication can then be assessed with PCR-based detection of viral DNA, p24 enzyme-linked immunosorbent assay (ELISA) for viral replication, flow cytometry for T-cell subpopulations, and semiquantitative assays for virus isolation.

Given the data that can be obtained about the effects of various antiviral compounds in vivo, this SCID-hu model may provide a useful means for the evaluation of preclinical lead compounds prior to movement into the clinic.

MATERIALS AND METHODS

Stock preparation of PHA-activated PBMCs. Standardized stocks of phytohemagglutinin (PHA)-activated peripheral blood mononuclear cells (PBMCs) were generated from buffy coats of human cells (obtained from the Stanford Blood Bank). Approximately 35 ml of the buffy coat cell suspension was mixed with an equal volume of phosphate-buffered saline (PBS) containing 8 U of heparin per ml, divided into aliquots in 50-ml conical centrifuge tubes, underlayered with Ficoll-Hypaque (Histopaque; Sigma) at a ratio of 3:1, and centrifuged at 500 × *g* for 30 min. The interface cells were harvested, washed twice with PBS, and counted. For activation with PHA, the cells were incubated at a concentration of 2×10^6 cells per ml in Iscove's modified Dulbecco's medium (IMDM; Gibco) containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U of penicillin-streptomycin per ml, and 1 μg of PHA (Sigma) per ml. After 2 days of incubation in a humidified 5% CO_2 atmosphere, the cells were harvested, divided into aliquots of 10^7 cells per vial, and frozen for future use in liquid N_2.

Stock preparation of HIV-1 isolates. All procedures with infectious isolates or molecular clones of HIV-1 were carried out in a Biosafety Level 3 facility or in a restricted animal barrier facility under guidelines for a Biosafety Level 3 facility. Plasmids containing the molecularly cloned viruses JR-CSF (16) and NL4-3 (1) were obtained from the repository of the National Institutes of Health. The clinical isolates EW and JD were derived from HIV-1-seropositive patients. HIV-1 isolates were expanded in PHA-activated PBMCs from healthy donors. Virus seed stocks from clinical isolates were obtained after cocultivation of patient-derived PHA-activated PBMCs with PHA-activated PBMCs from healthy donors. Seed stocks of molecular clones were obtained by electroporation of 25 μg of HIV-1 DNA per 5×10^6 cells (Bio-Rad Gene Pulser) at 960 μFD and 280 V.

Working stocks of virus were prepared by infecting 10^8 PHA-activated PBMCs with 2×10^5 $TCID_{50}$s of viral inoculum in a volume of 5 ml. After 2 h at 37°C, the cells were diluted to a density of 2×10^6 to 3×10^6 per ml in IMDM containing 10% FCS, 2 mM L-glutamine (Gibco), 100 U of penicillin-streptomycin (Gibco) per ml, and interleukin-2 (IL-2; Cellular Products) at 50 U/ml (viral culture medium). On day 2 the cells were pelleted and fresh medium was added, and the supernatant was collected 24 h later. This cycle was repeated on days 4 to 8, with the addition of fresh medium containing IL-2 after each harvest. Supernatants were divided into aliquots, directly frozen, and stored in liquid nitrogen. Samples were analyzed for p24 content by ELISA and for infectious virus titer by $TCID_{50}$ assay.

$TCID_{50}$ assay for HIV-1. PHA-activated PBMCs were thawed from a standard stock, cultured overnight in viral culture medium, and plated into 4 replicate wells of a 96-well plate (10^5 cells in 25 μl per well for each dilution of virus to be assayed). In a separate series of wells, 5- to 10-fold serial dilutions of virus were prepared in medium containing 10 μg of Polybrene (Sigma) per ml. An equal volume (25 μl) of each dilution was added to the quadruplicate wells of PHA-activated PBMCs. After 2 h at 37°C, 200 μl of viral culture medium was added to each well and the plates were maintained at 37°C in a humidified 5% CO_2 atmosphere. After 7 days, the plates were centrifuged at 400 × *g* for 5 min, the supernatants were carefully removed from each well, and the pellets were lysed with 100 μl of p24 lysis buffer (see below) and assayed for p24 antigen content at the appropriate dilution. The $TCID_{50}$ is expressed as the reciprocal of the dilution at which 50% of the wells contained detectable p24 and specifies the number of infectious doses per 25 μl.

p24 ELISA. For the quantitation of HIV-1 p24 within cells, 1×10^5 to 5×10^6 cells were lysed overnight at 4°C in 100 μl of p24 lysing buffer (containing 1% Triton X-100, 0.5% deoxycholate, 5 mM EDTA, 25 mM Tris HCl, 250 mM NaCl, and 1% aprotinin). In the case of virus supernatants, 10% Triton X-100 was added to a final concentration of 1%. These preparations were then transferred into a quantitative ELISA (Dupont) by using HXB2-infected H9 cells to generate a standard curve. Results are reported as the number of picograms of p24 per 10^6 cells or, in the case of $TCID_{50}$ determinations, as detectable or undetectable.

Construction of SCID-hu Thy/Liv mice. Homozygous C.B-17 *scid*/*scid* mice (SCID) were bred at SyStemix and were treated prophylactically with trimethoprin-sulfamethoxasole to prevent opportunistic infection with *Pneumocystis carinii* (19). For surgical procedures, mice (8 weeks old) were anesthetized with 5% ketamine–2.5% xylazine (10 μl/g of body weight), which was injected intramuscularly in sterile PBS. Implantation of fragments of human fetal liver and human fetal thymus to create SCID-hu Thy/Liv mice was carried out as described previously (22). Cohorts of animals (n = 20 to 50) were produced with tissues from the same donor. At 12 to 20 weeks posttransplantation, randomly chosen animals from each cohort (20% of the total) were examined under general anesthesia to evaluate the growth of the Thy/Liv implant. If the size of the implants was greater than or equal to approximately 30 mm^3 (e.g., 3 by 3 by 3 mm in dimension), the entire cohort was then entered into the antiviral dosing experiments. These conditions pertained in approximately 80 to 90% of the cohorts produced.

Preparation of drugs for administration to SCID-hu mice. AZT was obtained from Sigma or from the repository of the National Institutes of Health, dideoxyinosine (ddI) was obtained from the repository of the National Institutes of Health, and nevirapine and its vehicle, 1% hydroxypropylmethyl (HPM) cellulose, were obtained from Sandoz. AZT solutions were prepared in water, allowed to stand overnight at 4°C, placed in a 37°C water bath for further dissolution if necessary, and then filtered through a 0.2-μm-pore-size filter, divided into aliquots, and stored at 4°C until use. ddI solutions were prepared by dissolving the compound in filtered (pore size, 0.2 μm) water adjusted to approximately pH 9.5; aliquots were stored at 4°C until use. Nevirapine was synthesized as described previously (11, 23), and for oral administration, it was dissolved at a concentration of 20 mg/ml in a 1% (wt/vol) suspension of HPM cellulose in tap water. The suspension was stored at room temperature. Each aliquot was vortexed for several minutes before administration to animals. As indicated in the text, various routes of administration were used, including oral (by gavage), intraperitoneal, subcutaneous (by injection or by minipump), and intravenous.

HIV-1 infection of SCID-hu Thy/Liv mice. Although infection of the Thy/Liv implant can be achieved at low frequency after intravenous or intraperitoneal inoculation of virus, the most reliable means of infecting mice has proved to be direct intrathymic injection of small volumes of standardized viral stocks (resulting in infection of essentially all animals). These infections were carried out in a restricted animal barrier facility under guidelines for Biosafety Level 3 facilities (e.g., with mask, eye covering, and gown). The mice were placed under general anesthesia, and to maximize visual and manual access, teams of three operators worked side-by-side on a bench top. One operator shaved the left flank of the mouse and, with a 1.0- to 1.5-cm excision, exposed the left kidney carrying the Thy/Liv implant. The second operator then gently immobilized the kidney with forceps and, using a 1-ml tuberculin syringe filled with India ink and equipped with a 27-gauge by 5/8-in. (1.6-cm), sharp needle, marked an opening through the fibrous kidney capsule. By using the India ink as a guide, the Thy/Liv implant was injected with 25 to 50 μl of virus stock in one to three places by using a 30-gauge by 1/2-in. (1.3-cm) blunt needle. A third operator repaired the incision by approximating the peritoneal lining with one stitch and using one to two staples to close the skin.

Levels of nevirapine in plasma. Frozen heparinized blood samples were thawed and centrifuged, and 0.5-ml aliquots in borate buffer (pH 9.3) were extracted with toluene. The toluene phase was then extracted with a 1:1 mixture of 0.25 M HCl and methanol; the aqueous layer was taken to dryness and was dissolved in 200 μl of a 6:4 methanol-water mixture. The high-performance liquid chromatography column was a Merck Lichrospher RP 18 column (250 by 4 mm) with a 4-by-4-mm precolumn. Elution was with 29% acetonitrile–71% 10 mM phosphate buffer (pH 7) and was done isocratically at 1 ml/min. The compound was detected by UV absorption at 234 nm. Nevirapine eluted as a single peak after 8.2 min and was quantified by area integration. Concentrations of nevirapine in blood were calculated from a standard curve of extracted blood samples spiked with nevirapine. The calibration curves were linear, with an r^2 value of 0.999; the detection limit was 8 ng/ml.

Collection of human graft samples from SCID-hu mice. After the mice were killed by CO_2 inhalation, the transplanted human Thy/Liv implants were removed by surgical excision and were transferred to 24-mm^2 tissue culture wells containing sterile PBS–2% FCS at 4°C. A single-cell suspension was made by placing the tissue into a sterile Nitex (Tetko, Inc.) bag, and while the open end of the bag was closed with a pair of forceps, the tissue was submerged into PBS–2% FCS and gently ground between the Nitex layers. The cells were counted on a Coulter counter and were divided into aliquots containing the appropriate numbers of cells for each assay. For DNA PCR, pellets (10^5 cells) were processed immediately by the direct lysis PCR method (below) or were

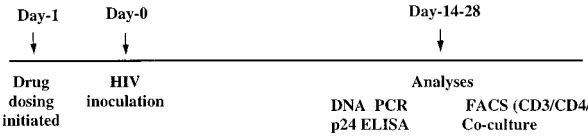

FIG. 1. Standardized protocol for evaluation of antiviral compounds in the SCID-hu mouse. See text for details.

stored at −80°C. For the p24 ELISA, pellets (3×10^6 to 5×10^6 cells) were resuspended in 400 μl of p24 lysis buffer, rotated overnight at 4°C, and stored at −20°C prior to analysis. For coculture analysis, log order dilutions of thymocytes (10^5, 10^4, 10^3, and 10^2) were cocultured with 10^5 PHA-activated PBMCs for 5 days; the cell cultures were then analyzed for the presence of HIV-1 p24. For fluorescence-activated cell sorter (FACS) analysis, 10^6 cells were resuspended in 1 ml of PBS–2% FCS and were analyzed on the same day.

DNA PCR. Infection of human cells in the transplanted Thy/Liv graft was assessed by PCR amplification with primers specific for the conserved U5-*gag* region of the HIV-1 genome (primers SK 431 and SK 145; Cetus) and human β-globin as a control for human DNA. Pellets (10^3 cells) were resuspended in 100 μl of lysis buffer containing 1% Nonidet P-40, 1% Tween 20, 2.5 mM $MgCl_2$, 5 mM KCl, and 10 μg of proteinase K per sample. The samples were vortexed, pulse spun, incubated at 60°C for at least 1 h, heated at 95°C for 10 min to inactivate the proteinase K, vortexed, pulse spun again, and assayed by a standard PCR assay as described previously (20). PCR was continued for 40 cycles (first cycle, 95°C for 6 min, 60°C for 2 min, and 72°C for 1.5 min; second cycle, 95°C for 2 min, 60°C for 2 min, and 72°C for 1.5 min; third cycle, 95°C for 1 min, 60°C for 1 min, 72°C for 1.5 min; final extension, 72°C for 10 min and 27°C for 8 h). The amplified products were run on a 3% agarose gel (2% NuSieve, 1% Seakem) by using either BioMarker Low or *Bst*EII-digested bacteriophage lambda arms as molecular weight standards. Gels were soaked for 30 to 40 min in ethidium bromide and were photographed. This assay was able to detect 1 HIV-1-positive cell per 100 input cells.

FACS analysis for thymocyte depletion. A total of 10^6 pelleted cells were resuspended in 50 μl of monoclonal antibody cocktail containing CD4-fluorescein isothiocyanate and CD8-phycoerythrin (Becton Dickinson) and CD3-tricolor (Caltag), with isotype controls prepared for each antibody mixture. After a 45-min incubation in the dark, the cells were washed three times, resuspended in 150 μl of 2% FCS–PBS, and transferred to 1.5-ml FACS tubes containing 50 μl of 4% paraformaldehyde. The fixed cells were removed from the Biosafety Level 3 laboratory and analyzed on a FACScan instrument (Becton Dickinson). Data for a total of 10,000 events were collected, and the percentages of $CD4^+$ and/or $CD8^+$ thymocytes were determined after gating on a lymphoid population identified by forward-scatter and side-scatter characteristics.

Data analysis. In all experiments, five to eight control mice were infected with HIV-1 and not treated with antiviral compounds and one to five control mice were not infected with HIV-1 and not treated. In the experimental groups, five to eight mice per group were infected and treated with various doses of antiviral compound. If the results of the initial experiments were consistent with the possibility of drug toxicity (e.g., low cell yields from the Thy/Liv implants at the termination of the experiment), additional animals were dosed with drug alone to assess toxicity specifically. The p24 results for the drug-treated groups in a given experiment are expressed as a percentage of the value for the control, in which the control value is the mean amount of p24 produced per 10^6 cells in the infected, untreated group. These results are expressed as the mean ± standard error for a given group. Statistical analyses were carried out by a nonparametric method by using the Mann-Whitney U test.

RESULTS

Development of a standardized SCID-hu assay. The SCID-hu Thy/Liv model has been found to sustain a time-dependent increase in the signs of HIV-1 replication (as measured by p24 ELISA, in situ hybridization, and PCR) (20, 21). After infection with some but not all virus isolates, thymocyte depletion has been observed within 3 to 5 weeks (2, 3, 14, 25). These observations served as a guide for the development and optimization of a protocol for the systematic analysis of antiviral compounds (Fig. 1). In this protocol, human fetal thymus and liver are implanted into the SCID mouse and allowed to grow over the next 3 to 4 months into a conjoint Thy/Liv organ. At 1 to 5 days before or 1 to 7 days after the inoculation of this organ with virus, test compounds are administered by a variety of routes (intraperitoneal, intravenous, subcutaneous, or oral or by minipump) by using a dosing schedule determined by prior pharmacodynamic analyses in rodents. On day 0, titered inocula of HIV-1 are delivered by direct injection into the implant. Antiviral compounds are administered for an additional period of time (2 to 4 weeks), at which point endpoint analyses are performed: PCR for viral proviral DNA, flow cytometry for the evaluation of cell subpopulations, p24 ELISA, and virus isolation by cocultivation.

The results generated in this protocol are significantly affected by two variables. First, individual virus isolates demonstrate various potentials for replication in vivo (14) and differential susceptibilities to given antiviral compounds. Since standardized procedures are used to create the individual virus stocks (see Materials and Methods), these variations are likely not reflective of lot-to-lot variations but of intrinsic biologic differences between the virus isolates instead. To minimize the impact of this variability, compounds are tested against two or more virus isolates, including an infectious molecular clone which grows on human T-cell lines (NL4-3) (1), an infectious molecular clone which has not been passaged for a long period of time in vitro and which does not grow on human T-cell lines (JR-CSF) (16), and several early-passage uncloned isolates from patients which are tropic for both T and myeloid cells (EW and JD). The use of a panel as opposed to one HIV-1 isolate makes it less likely that antiviral compounds may appear efficacious (or not) because of isolate-specific effects.

A second variable in the SCID-hu model rests upon donor-to-donor differences in the ability of the human Thy/Liv implant to sustain viral replication in vivo (as measured by the absolute amount of p24 made per 10^6 cells), not dissimilar to donor-dependent differences in viral replication observed on lots of PHA-activated PBMCs in vitro (29). To eliminate this variable from the analysis, experiments are generally conducted with SCID-hu mice carrying Thy/Liv implants from the same fetal donor. Since implants in 20 to 50 animals can be generated from a single donor, experiments can be designed with two to six separate groups of four to eight animals each, not including infected, untreated and uninfected, untreated control groups. When the p24 results are then expressed as a percentage of the value for the control (see Data analysis in the Material and Methods section), data from multiple experiments with large numbers of animals can be combined. As an example, Fig. 2 depicts the results for 75 animals from five separate cohorts of SCID-hu mice, with the implants in each cohort made with a different donor; Fig. 3 and 4 represent the results of dose-response studies carried out with replicate cohorts of animals with implants made from different donors.

HIV-1 infection of the Thy/Liv model. In the absence of virus challenge, normal human thymopoiesis is supported by the Thy/Liv model (17, 22, 27, 28). After infection with virus isolates such as NL4-3 or EW, a time-dependent increase in the level of virus replication was observed. By the p24 ELISA, virus production was detectable by day 10 and peaked on days 14 to 21 (Fig. 2A). As measured by a DNA PCR assay, virus was undetectable at day 3 but was found in 100% of Thy/Liv implants by day 14 (Fig. 2B). Similar kinetics were observed when infectious virus particles were quantitated by a coculture assay (data not shown).

After 14 to 21 days, the absolute levels of virus replication dropped as a result of HIV-1-mediated thymocyte depletion (2, 3, 14, 25, 26). Thus, in the setting of NL4-3 or EW infection, the $CD4^+$ $CD8^+$ ("cortical") thymocyte population steadily decreased until it was virtually nondetectable by day 35 to 42. At the same time, there was an inversion of the CD4/CD8 ratio. In the case of some isolates (e.g., NL4-3), there was also a time-dependent decrease in the representation of $CD3^-$ $CD4^+$ $CD8^-$ intrathymic T progenitor cells. In practice, this

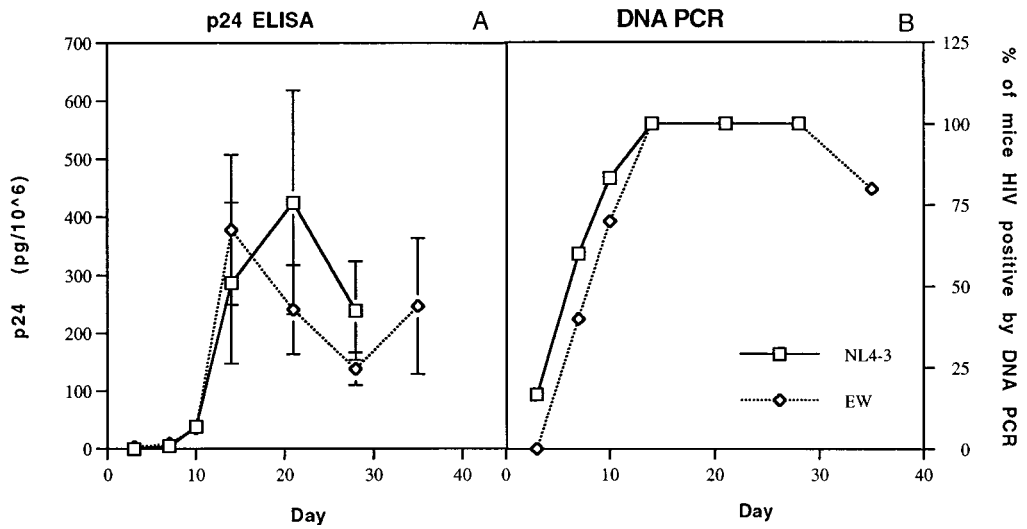

FIG. 2. Time course of infection of SCID-hu Thy/Liv mouse with EW or NL4-3. Cell-associated HIV-1 p24 production and DNA PCR results were evaluated over a time course of 0 to 35 days following intrathymic inoculation of EW or NL4-3. p24 was evaluated for each animal as the number of picograms per 10^6 cells, and average values for 75 animals from five experiments are reported as the mean ± standard error. A total of 100 thymocytes from individual animals were assayed by DNA PCR for evidence of the HIV-1 genome. Results are reported as the percentage of positive animals and represent cumulative data for four to five experiments with each virus. For EW, there was one experiment with five mice per group that was terminated at days 3, 7, 10, and 14; one experiment with five mice per group that was terminated at days 7, 10, 14, and 21; one experiment with five mice per group that was terminated at days 14 and 28; one experiment with five mice per group that was terminated at days 14, 21, 28, and 35; and one experiment with five mice per group that was terminated at day 28. For NL4-3, there was one experiment with five mice per group that was terminated on days 1 and 14; one experiment with five to six mice per group that was terminated on days 7, 14, 21, and 28; and two experiments with five mice per group that was terminated on days 14 and 28.

phenomenon dictates that endpoint analyses of the antiviral effect be carried out 2 to 3 weeks postinoculation of virus, before such time as any cellular depletion occurs.

Dose-response studies with AZT and ddI. The observations presented above provide a baseline against which to evaluate the preclinical efficacies of antiviral compounds. Preliminary studies in the Thy/Liv system were carried out with licensed antiviral agents, e.g., AZT (Retrovir) and ddI. When tested in vitro in PHA-activated PBMCs, AZT was found to be equally potent against both EW and NL4-3, with 50% inhibitory concentrations of 0.043 and 0.015 μM, respectively. Both isolates were also equally susceptible to in vitro inhibition by ddI. The relative potency of ddI was, however, 2 to 3 log orders less than that of AZT (with 50% inhibitory concentrations of 1.58 μM for NL4-3 and 1.23 μM for EW).

In contrast, when tested in the SCID-hu Thy/Liv system, AZT and ddI appeared to be at least equipotent; additionally, ddI showed about 40 times greater activity against EW than against NL4-3. Thus, various doses of AZT and ddI were administered to cohorts of animals infected with either NL4-3 or EW. As shown in Fig. 3, there was a dose-dependent reduction in the level of p24 at the 14-day endpoint. With AZT, 50% inhibitory dosages approximated 20 mg/kg/day for NL4-3 (Fig. 3A) and 11 mg/kg/day for EW (Fig. 3B); with ddI, the corresponding 50% inhibitory dosages were 65 mg/kg/day for NL4-3 (Fig. 3C) and 1.5 mg/kg/day for EW (Fig. 3D). Statistically significant reductions (Mann-Whitney U test) were also observed in repeat experiments when ddI was administered for as long as 7 days after infection with either EW or NL4-3; postexposure administration of AZT at this time point did not result in the suppression of virus replication (data not shown).

Even in the highest dosage ranges of AZT (40 to 180 mg/kg/day), most animals infected with EW or NL4-3 remained DNA PCR positive for HIV-1. Similar results were observed when animals were infected with NL4-3 and treated with ddI. In contrast, in two experiments with ddI against EW, 0 of 14 animals given 20 mg/kg/day were DNA PCR positive, 2 of 15 animals given 6.7 mg/kg/day were positive, and 3 of 8 animals given 2.2 mg/kg/day were positive (data not shown).

Thymocyte depletion was significantly suppressed at 4 weeks when ddI (50 mg/kg/day) was administered to animals infected with either NL4-3 or EW (Fig. 4). AZT administration, on the other hand, suppressed thymocyte depletion at the same time period when given at a dosage of 150 mg/kg/day against NL4-3 but did not suppress thymocyte depletion when it was given at a dosage of 95 mg/kg/day against EW.

Dose-response studies with nevirapine. Whereas AZT and ddI need metabolic activation (phosphorylation) to exert their antiviral effects, nevirapine does not. It was therefore of interest to determine whether the levels of this nonnucleoside reverse transcriptase inhibitor in blood correlated with antiviral efficacy in vivo. Oral treatment of HIV-1-infected patients with 12.5 mg of nevirapine per day (ca. 0.2 mg/kg/day) or greater causes a rapid decline in serum p24 antigen levels (4, 12). A single oral dose of 12.5 mg of nevirapine results in an area under the concentration-time curve from time zero to infinity ($AUC_{0-\infty}$) of 4.7 to 10.2 μg · h/ml in humans (5, 13).

To obtain statistically significant (70 to 80%) inhibition of p24 production in SCID-hu mice infected with HIV-1 isolates NL4-3 and EW, dosages in the range of 10 to 25 mg/kg/day (oral, twice daily) were required (Fig. 5). A single oral dose of 10 mg/kg of nevirapine gave an $AUC_{0-\infty}$ of 2.1 μg · h/ml (maximum concentration of drug in serum, 1 μg/ml; time to maximum concentration of drug in serum, 0.5 h) (data not shown). Increasing the dosage of nevirapine to 100 mg/kg/day gave 99% inhibition of p24 production, while the $AUC_{0-\infty}$ for a single dose increased to 83.7 μg · h/ml.

DISCUSSION

Methods have previously been devised for the efficient implantation of human fetal thymus and liver fragments into

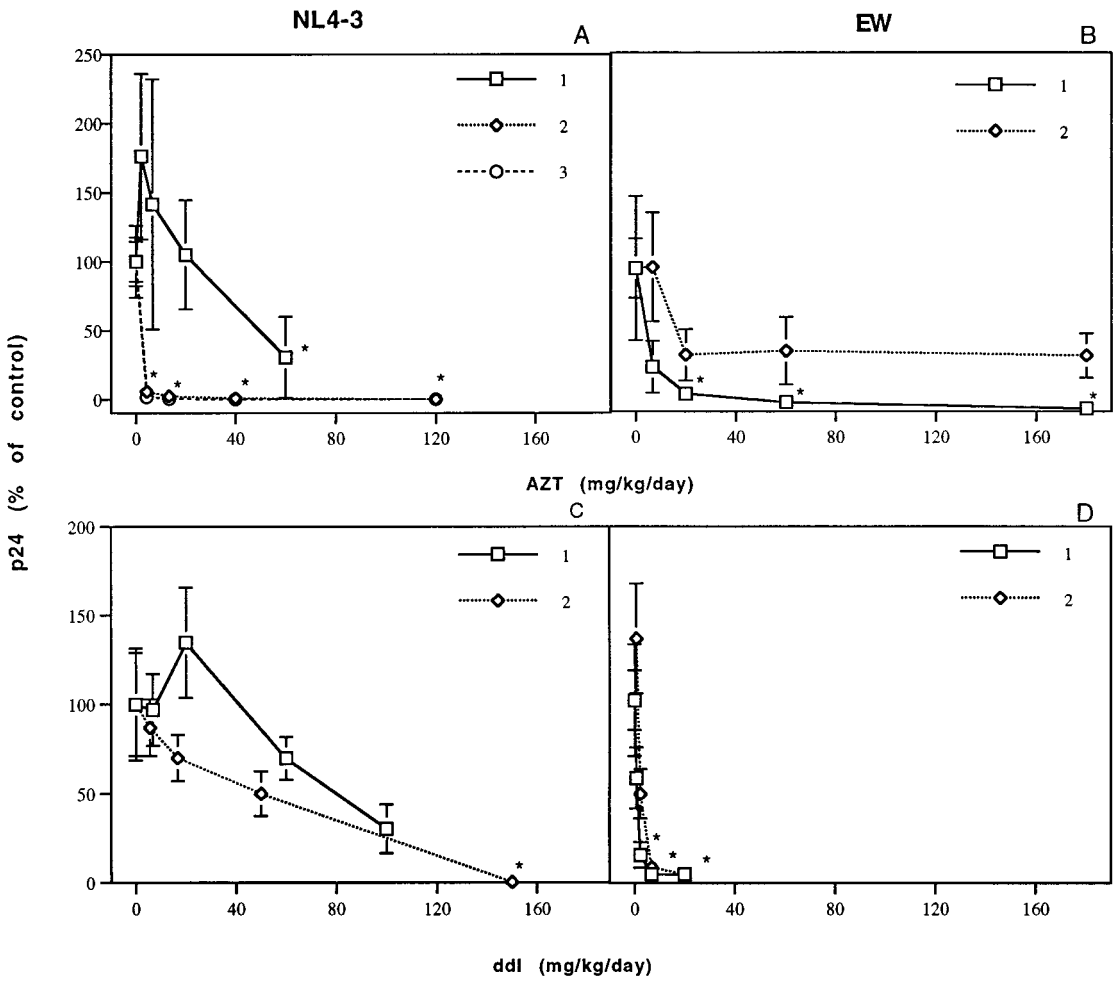

FIG. 3. Dose-response studies with AZT or ddI in SCID-hu Thy/Liv mice infected with EW or NL4-3. AZT and ddI were administered twice daily (AZT by gavage; ddI by intraperitoneal injection) by the protocol shown in Fig. 1 and as described in the text. p24 levels are expressed as the mean percentage of the level in the control at each dose, with standard errors indicated by bars. Datum points in which p24 production is significantly different (by Mann-Whitney U test) from that for the infected, untreated control group are indicated by an asterisk. Each line represents a separate experiment with mice with implants prepared from a different fetal tissue donor.

SCID mice to create SCID-hu Thy/Liv mice. This human implant system is both structurally and functionally similar to the normal human thymus (17, 22, 27, 28) and permissive for HIV-1 replication (2, 3, 14, 21, 25). A standardized protocol has now been devised for the use of this model in the preclinical evaluation of antiviral compounds against HIV-1. Drugs can be administered by various routes (intraperitoneal, intravenous, subcutaneous, or oral or by minipump) prior to infection (see also the companion report by Datema et al. [6]) and continued for a 2- to 4-week time period postinfection. Animals are challenged with titered inocula of standardized stocks of various HIV-1 isolates. Endpoint analyses include a quantitative measure of viral protein by p24 ELISA, a semiquantitative detection of proviral DNA by PCR, an assessment of the effects on various human thymocyte subpopulations by flow cytometry, and quantitation and isolation of replication-competent virus by cocultivation under limiting dilution.

This protocol has been in use for 3 years, during which time implants in a total of 3,980 animals derived from 225 human fetal donors have been used in the evaluation of 14 different compounds. A total of 119 of 121 (or >98%) of the experiments have been deemed technically sound, with problems arising in two experiments because of poor-quality virus stocks.

As shown here, the SCID-hu Thy/Liv model reveals the dose-dependent anti-HIV-1 activities of the antiviral compounds AZT, ddI, and nevirapine in vivo. A dose-dependent reduction in the virus load can also be obtained with other anti-HIV-1 drugs, including a bicyclam (6) and a nonimmunosuppressive cyclosporin analog (23a). Given the number of animals (n = 5 to 8) in a given dosing group, it is possible to assign effective dose ranges against HIV-1 with statistical confidence. Among animal models for HIV-1, the one described here is unique; nonhuman primate models cannot provide dosing groups of such size, and hence, studies with such models cannot achieve the same statistical power as that obtained with the SCID-hu model; additionally, some of the compounds studied (e.g., nevirapine) are not active against retroviruses which replicate in other species (e.g., murine leukemia virus, feline immunodeficiency virus, and simian immunodeficiency virus) (7).

Three observations arise from this initial set of preclinical analyses. First, the relative potencies of AZT and ddI are dramatically different whether they are studied in vitro or in vivo. In vitro, AZT appears to be several log orders more potent than ddI against EW and NL4-3. In vivo, the compounds are approximately equipotent against both viruses; in

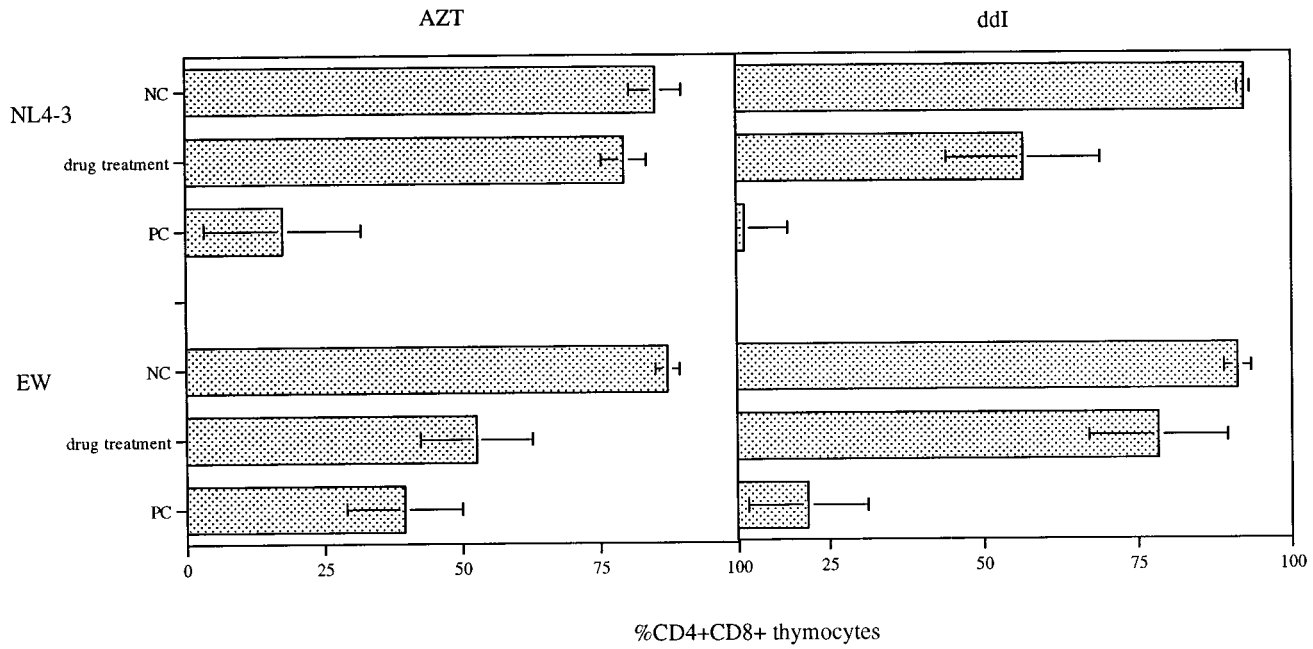

FIG. 4. Studies of thymocyte depletion in SCID-hu Thy/Liv treated with AZT or ddI. Mice infected with NL4-3 were treated with either 150 mg of AZT per kg/day or 50 mg of DDI per kg/day; those infected with EW were treated with either 95 mg of AZT per kg/day or 50 mg of ddI per kg/day. The drugs were administered as described in the legend to Fig. 3. Noninfected controls (NC) and infected, untreated controls (PC) were included in each experiment. FACS analyses were conducted at 4 weeks postinfection. Data reflect the results of three experiments.

fact, as judged by postexposure efficacy and by the larger fraction of animals negative for virus by DNA PCR when high doses were given preexposure, ddI appears to be even more potent. This may be due to the fact that ddI is more efficiently triphosphorylated than AZT in resting cells (10), the prevalent cell cycle phase found in vivo; alternatively, ddI may be selectively active within cell subpopulations that are present in vivo but that can be grown only with difficulty in vitro (e.g., macrophages). In either case, the apparent equipotence of AZT and ddI found in the SCID-hu Thy/Liv model is more consistent with the results obtained in human clinical trials (24), underscoring the fact that in vitro studies may provide inaccurate relationships about antiviral activity in vivo. In the case of nevirapine, which does not require triphosphorylation for its activity in vivo, the SCID-hu Thy/Liv model would also have predicted the antiviral efficacy at $AUC_{0-\infty}$ values that are readily obtained in humans.

Second, the replication of different HIV-1 isolates in the SCID-hu Thy/Liv model was suppressed by antiviral compounds in a differential manner that would have not been

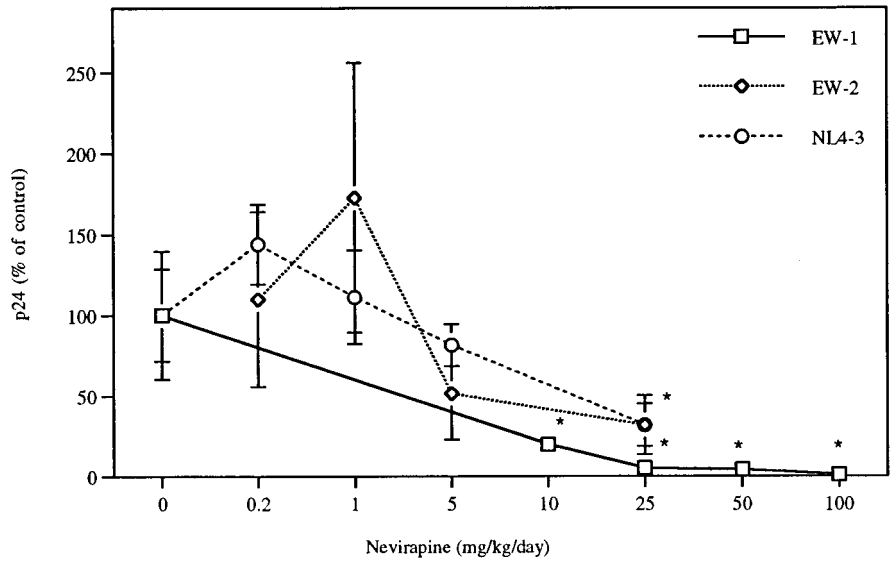

FIG. 5. Dose-response studies with nevirapine in SCID-hu Thy/Liv mice infected with EW and/or NL4-3. The drug was administered twice daily by gavage. Data are presented as described in the legend to Fig. 3. Two separate experiments were conducted with EW; one was conducted with NL4-3.

predictable on the basis of tests in vitro. Thus, EW and NL4-3 showed roughly equivalent susceptibilities to ddI in vitro (50% inhibitory concentration = 1.23 to 1.58 μM) but very different responses in vivo (50% infective dosage = 1.5 mg/kg/day for EW; 50% infective dosage = 65 mg/kg/day for NL4-3). Possibly, these differences are based on yet uncharacterized attributes of the respective virus stocks (e.g., with respect to cell tropism in vivo or the ability to generate drug-resistant variants). Absent knowledge about the molecular bases of these differences, these observations emphasize the need to use a panel of virus isolates in the preclinical evaluation of any antiviral compound.

Third, even though the Thy/Liv implants appeared to be grossly equivalent from one cohort of animals to another, an uneven, donor-dependent capacity to support the replication of a given, standardized virus stock was revealed. This variation was best reflected at the 2-week endpoint by the mean p24 levels from the infected, untreated positive control animals, all of which were constructed with tissue from a single human donor in a single experiment. After inoculation with the same NL4-3 stock, for example, the mean p24 levels for groups of animals with implants prepared from single donors ranged from 47 to 1,747 pg/10^6 cells; for EW, the range was 53 to 1,076 pg/10^6 cells. When the results of a given experiment are expressed as a percentage of the value for the control, variation in the range of absolute p24 levels was normalized and the data sets were comparable between experiments (with implants prepared by using different donors). This observation suggests that preclinical testing of antiviral compounds in vivo is facilitated by the ability to infect animals with identical genetic backgrounds.

It is also notable that the current SCID-hu model can be used to explore alternative treatment regimens. In other studies (6), this model has proved to be amenable to the evaluation of variable dosing routes (e.g., subcutaneous versus intraperitoneal versus continuous administration by minipump). Additionally, the large cohort sizes and the reproducibility of the model permit statistical evaluation of combination therapies against HIV-1.

In sum, the SCID-hu Thy/Liv model is usable as a reliable preclinical animal model for the evaluation of the anti-HIV-1 efficacies of various compounds. It is the first animal model in which the action of candidate anti-HIV-1 compounds can be tested within the setting of HIV-1-infected human target organs. As such, it may help to guide the selection of effective drugs to treat HIV-1 disease in humans.

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