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Potent Anti-R5 Human Immunodeficiency Virus Type 1 Effects of a CCR5 Antagonist, AK602/ONO4128/GW873140, in a Novel Human Peripheral Blood Mononuclear Cell Nonobese Diabetic-SCID, Interleukin-2 Receptor γ -Chain-Knocked-Out AIDS Mouse Model

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We established human peripheral blood mononuclear cell (PBMC)-transplanted R5 human immunodeficiency virus type 1 isolate JR-FL (HIV-1_{JR-FL})-infected, nonobese diabetic-SCID, interleukin 2 receptor γ -chain-knocked-out (NOG) mice, in which massive and systemic HIV-1 infection occurred. The susceptibility of the implanted PBMC to the infectivity and cytopathic effect of R5 HIV-1 appeared to stem from hyperactivation of the PBMC, which rapidly proliferated and expressed high levels of CCR5. When a novel spirodiketopiperazine-containing CCR5 inhibitor, AK602/ONO4128/GW873140 (molecular weight, 614), was administered to the NOG mice 1 day after R5 HIV-1 inoculation, the replication and cytopathic effects of R5 HIV-1 were significantly suppressed. In saline-treated mice ($n = 7$), the mean human CD4⁺/CD8⁺ cell ratio was 0.1 on day 16 after inoculation, while levels in mice ($n = 8$) administered AK602 had a mean value of 0.92, comparable to levels in uninfected mice ($n = 7$). The mean number of HIV-RNA copies in plasma in saline-treated mice were $\sim 10^6$ /ml on day 16, while levels in AK602-treated mice were 1.27×10^3 /ml ($P = 0.001$). AK602 also significantly suppressed the number of proviral DNA copies and serum p24 levels ($P = 0.001$). These data suggest that the present NOG mouse system should serve as a small-animal AIDS model and warrant that AK602 be further developed as a potential therapeutic for HIV-1 infection.

Highly active antiretroviral therapy has brought about a major impact on the AIDS epidemics in the industrially advanced nations (5, 22). However, eradication of human immunodeficiency virus type 1 (HIV-1) is thought to be currently impossible, due in part to the viral reservoirs remaining in blood and infected tissues (6). The limitation of antiviral therapy of AIDS is exacerbated by complicated regimens, the development of drug-resistant HIV-1 variants (11), and a number of inherent adverse effects (2, 31). Hence, the identification of new antiretroviral drugs that have unique mechanisms of action and produce no or minimal adverse effects remains an important therapeutic objective. In regard to development of potential anti-HIV therapies or vaccines, experimental animal models for AIDS which allow the determination of the possible efficacy of antiviral agents or vaccines have been sought since severe

combined immunodeficiency (SCID) mice engrafted with human fetal thymus, liver, or peripheral blood mononuclear cells (PBMC) were first exploited to examine antiretroviral agents (19, 25). However, a number of mouse models have suffered from false-positive and false-negative results in detecting or quantifying HIV-1 infection and replication and have required a large number of samples and mice for testing (25, 29).

In the present work, we established human PBMC-transplanted R5 HIV-1_{JR-FL}-infected, nonobese diabetic (NOD)-SCID, interleukin 2 receptor γ (IL-2R γ)-chain-knocked-out (NOG) mice, in which massive and systemic HIV-1 infection occurs, human CD4⁺/CD8⁺ cell ratios significantly decrease, and high levels of R5 HIV-1 viremia reaching as high as 10^6 copies/ml are achieved. Furthermore, we demonstrated that this unprecedented susceptibility of the implanted human PBMC to the infectivity and cytopathic effects of R5 HIV-1 infection stems from hyperactivation of the PBMC. Here, we also report a novel small nonpeptide CCR5 antagonist, AK602/ONO4128/GW873140, which exerts potent anti-HIV-1 activity in vitro against laboratory and clinical strains of HIV-1, including highly multidrug-resistant (MDR) variants.

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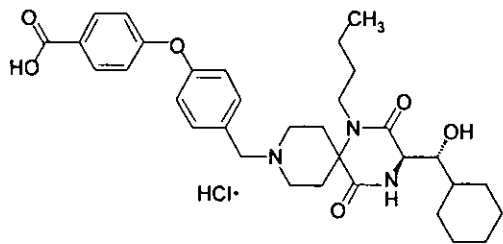


FIG. 1. Structure of AK602.

MATERIALS AND METHODS

Transplantation of human PBMC in NOG mice. NOD-SCID⁺ (NOG) mice (10, 33) were maintained in the Central Institute for Experimental Animals (Kawasaki, Japan). Mice were 4 to 6 weeks old at the time of transfer of human PBMC. The human PBMC-transplanted NOG (hu-PBMC-NOG) mice were generated by methods previously described (23, 24). Briefly, PBMC (10^7) were freshly prepared from heparinized blood of a single healthy HIV-1-seronegative donor by Ficoll-Hypaque density gradient centrifugation, resuspended in RPMI 1640-based culture medium (0.5 ml), and infused intraperitoneally to each mouse. The experimental protocol was approved by the Ethics Review Committees for Animal Experimentation of the participating institutions.

Assay for proliferation and CCR5 expression of transplanted human PBMC recovered from hu-PBMC-NOG mice. Freshly isolated human PBMC (2×10^7 cells/ml) were incubated in phosphate-buffered saline (PBS) containing 10 μ M 5-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, Oreg.) for 15 min at 37°C for CFSE labeling as previously described by Lyons (16), washed, and resuspended in RPMI 1640. One part of the labeled PBMC preparation was intraperitoneally injected (10^7 PBMC) to each NOG mouse, and human PBMC were recovered from peritoneal lavages and spleen. The other part of the preparation was immediately stimulated with 10 μ g of phytohemagglutinin (PHA)/ml, cultured, and harvested. PBMC samples thus obtained were labeled with phycoerythrin (PE)-conjugated anti-CCR5 monoclonal antibody 3A9 or peridinin chlorophyll protein-conjugated anti-HLA-DR antibody (BD Pharmingen, San Diego, Calif.) and subjected to flow cytometric analysis with a Becton Dickinson FACScan cytometer; the data were analyzed by Cell Quest software (Becton Dickinson, Franklin Lakes, N.J.). A quantitative fluorescence-activated cell sorting (FACS) assay that relies on a series of precalibrated beads that bind to a fixed number of mouse immunoglobulin G molecules (Quantum Simply Cellular Kit; Sigma, Saint Louis, Mo.) to determine the absolute number of CCR5s on the cell surface was also conducted according to the manufacturer's instructions (15).

Cells and viruses. The HeLa-CD4-LTR- β -gal indicator cell line expressing human CCR5 (CCR5⁺ MAGI) (18), a kind gift from Yosuke Maeda, was used for the present study. 293T cells (a human embryonic kidney cell line) were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (FCS) and antibiotics and used for transfection of DNA plasmid containing the R5 HIV-1_{JR-FL} genome (13). PBMC isolated from HIV-1-seronegative individuals were cultured with 10% FCS and antibiotics with 10 μ g of PHA/ml for 3 days prior to anti-HIV-1 activity assay in vitro (PHA-PBMC). A panel of HIV-1 strains was employed for the drug susceptibility attempt: HIV-1_{BR-1} (7), HIV-1_{JR-FL} (13), HIV-1_{NI-4.3} (32), a wild-type HIV-1_{MOKW} isolated from a drug-naïve AIDS patient (17), and MDR primary HIV-1 (HIV-1_{MDR}) strain (HIV-1_{JSI} and HIV-1_{MM}) (35). All primary HIV-1 strains were passaged once or twice in PHA-PBMC cultures and the culture supernatants were stored at -80°C until use. Antiviral assays using PHA-PBMC were conducted as previously reported (12, 17, 35).

Antiviral agents and assay for inhibition of R5 HIV-1 infectivity and replication. A series of different spirodiketopiperazine (SDP) derivatives were newly designed, synthesized, and tested for their activity against in vitro infectivity and replication of R5 HIV-1 as previously described (17). AK602 was chosen for this study based on its CCR5-specific, potent activity against R5 HIV-1. A method for the synthesis of AK602 will be published elsewhere. The structure of AK602 is illustrated in Fig. 1. An approved drug for therapy for HIV-1 infection, 2',3'-dideoxyinosine (ddI) (20, 21), was kindly provided by Ajinomoto Co., Inc. Tokyo, Japan. TAK779 and SCH-C were synthesized according to previously published data (1, 30). The MAGI assay using CCR5⁺ MAGI cells was conducted as previously described (17) with minor modifications. Briefly, CCR5⁺ MAGI cells were seeded in 96-well, flat-bottomed microculture plates (10^4 cells/well) for 24 h, exposed to 0.1 or 1 μ M AK602 for 30 min, washed three times, exposed to

R5 HIV-1 (100 50% tissue culture infectious doses) at various time points after AK602 removal, and cultured in Dulbecco's modified Eagle medium containing 15% FCS for 48 h. Following the removal of supernatants and lysis of the cells with PBS (100 μ l) containing 1% Triton X-100, a solution (100 μ l) containing 10 mM chlorophenol red- β -D-galactopyranoside, 2 mM MgCl₂, and 0.1 M K₂HPO₄ was added to each well; the mixture was incubated at room temperature in the dark for 30 min; and the optical density (wavelength, 570 nm) was measured with a microplate reader (Vmax, Molecular Devices, Sunnyvale, Calif.). All assays were performed in triplicate.

Pharmacokinetic analysis of AK602 in hu-PBMC-NOG mice. Pharmacokinetic analysis of AK602 in hu-PBMC-NOG mice was performed as previously described (28). In brief, plasma samples were collected periodically over 12 h, following a single AK602 administration at a dose of 60 mg/kg of body weight dissolved in 400 μ l of 4% hydroxypropyl- β cyclodextrin (HPBC). Each plasma sample (150 μ l) was centrifuged at 3,000 rpm for 10 min, and the supernatant was vacuum concentrated and injected into the high-performance liquid chromatography (HPLC) system. The eluent was monitored at 255 nm of UV, and the AK602 concentration in plasma was determined.

Determination of amounts of AK602 persistently bound to CCR5 in hu-PBMC-NOG mice. Blood samples were collected from the tail vein of each hu-PBMC-NOG mouse at various time points following a single intraperitoneal administration of AK602 at a dose of 60 mg/kg. PBMC were isolated by density gradient centrifugation and stained with fluorescein isothiocyanate-conjugated monoclonal antibody 45531 (R&D Systems, Minneapolis, Minn.) specific for the C-terminal half of the second extracellular loop (ECL2B) of CCR5 (15) known to be competitively replaced by SDP derivatives (17) or with PE-conjugated monoclonal antibody 3A9, which binds to the N-terminus extracellular domain of CCR5 (17). PBMC were then subjected to FACS analysis.

Treatment of R5 HIV-1-infected hu-PBMC-NOG mice with anti-HIV-1 agents. Sixteen days after PBMC infusion, the mice were bled from the tail vein, and three-color flow cytometric analysis was performed to confirm positive engraftment of human HLA, CD4, and CD8 antigens on the cells recovered. HIV-1_{JR-FL} (2,000 50% tissue culture infectious doses) was intraperitoneally inoculated to each mouse in which PBMC engraftment was confirmed. Twenty-four hours after the R5 HIV-1 inoculation, administration of AK602 (120 mg in 4% HPBC/kg/day, twice a day), ddI (50 mg in 4% HPBC/kg/day, twice a day), or saline was implemented and continued by day 16. On days 5 and 9 after the R5 HIV-1 inoculation, blood samples were collected from mouse tail veins for immunologic and virological monitoring (see below). On day 16, blood samples were collected by cardiocentesis, and the mice were sacrificed. The experimental protocol for the treatment is illustrated in Fig. 2.

Immunologic and virological monitoring. Human PBMC recovered from mice were subjected to immunologic and virological monitoring as previously described (23, 24). The CD4⁺:CD8⁺ cell ratios were determined by FACS analysis with PE-conjugated mouse anti-CD4 and peridinin chlorophyll protein-conjugated mouse anti-CD8 (BD Pharmingen) monoclonal antibodies. Determination of HIV-1 DNA copy numbers in recovered human PBMC was performed by real-time PCR assay with Taqman Master mixture (PE Biosystems) and HIV long terminal repeat-specific primers M667 (5'-GGC TAA CTA GGG AAC CCA CTG-3') and AA55 (5'-CTG CTA GAG ATT TTC CAC ACT GAC-3'). HIV-1-specific products were quantified with the ABI 7700 detection system (Applied Biosystems, Foster City, Calif.), and cell numbers were determined with the RAG-1 gene. The numbers of CD4⁺ cells were calculated based on the percentage of CD4⁺ values obtained from the FACS analysis of each test PBMC sample, and R5 HIV-1 proviral DNA copy numbers were expressed as copy numbers per 10^5 CD4⁺ cells. In some experiments, CD4⁺ and CD4⁻ cells were separated before real-time PCR assay with the rapid immunomagnetic CD4-positive cell isolation kit (Dynaheads M-450 CD4; Dynal Biotech, Inc. Lake

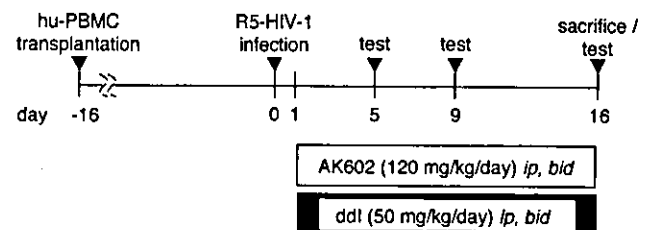


FIG. 2. Protocol for drug administration and immunological and virological monitoring.

Success, N.Y.). The amounts of p24 antigen in murine sera were determined using a fully automated chemiluminescent enzyme immunoassay system (I amipulse F; Fujirebio, Inc., Tokyo, Japan) as previously described (12). Plasma viral load was quantified with the AMPLICOR HIV-1 monitor test kit, version 1.5 (Roche Diagnostics, Branchburg, N.J.).

Statistical analyses. Nonparametric statistical analyses were performed by using the Mann-Whitney U test (Statview, version 5.0; Abacus Concepts, Berkeley, Calif.). The difference between viremia levels in two groups of mice was determined by the Wilcoxon rank sum test. For each mouse, the value of \log_{10} RNA copies was calculated, and the slope corresponding to the rate of increase per day was determined by simple linear regression for the days (5, 9, and 16) of blood collection. The resulting slopes for all mice in the untreated groups were compared to the slopes of mice in each of the other two groups.

RESULTS

Transplanted PBMC in hu-PBMC-NOG mice are intensely activated and express high levels of CCR5. When we examined the proliferation profile of PBMC stimulated with PHA *in vitro* by treatment with the vital dye CFSE, which allows the analysis of cell proliferation as the CFSE's fluorescence intensity is halved per each cell division, there was only a slight shift to the left in the flow cytometric profile on days 1 and 2 of culture (Fig. 3A). On day 4 of culture, a discrete shift to the left was identified, suggesting that the PHA-PBMC underwent up to four cycles of proliferation *in vitro* by day 4. In contrast, PBMC transplanted and recovered on day 2 had apparently undergone ~4 cycles of proliferation; by day 4, a majority of cells had undergone up to 10 cycles and beyond in proliferation (Fig. 3B). It was possible that the CFSE-negative and weakly CFSE-positive cells which accumulated on days 2 and 4 (Fig. 3B) were murine cells that engulfed and degraded CFSE. We therefore conducted experiments in which the cells with CFSE dilution were directly confirmed to be human CCR5-positive cells. As can be seen in Fig. 3C, when cells were recovered from the spleen of an NOG mouse into which CFSE-labeled PBMC had been transplanted and stained with monoclonal antibody 45531, which is specific for the C-terminal half of the second extracellular loop (ECL2B) of CCR5 (15), the majority of such human CCR5⁺ cells proved to be CFSE negative. We also examined the levels of cellular activation by the expression of HLA-DR on cell surface. The levels of HLA-DR expression in PBMC recovered from uninfected NOG mice 3 days after transplantation were much greater than those in 3-day-cultured PBMC following PHA stimulation (Fig. 3D). The fluorescence intensity in the same donor's PHA-PBMC examined on three different occasions was 21 ± 4 , while that of the PBMC recovered from mice was 91 ± 25 (Fig. 3D). When we further assessed the levels of CCR5 expression, the PBMC recovered from the mice on day 3 proved to be strongly positive for CCR5 (Fig. 3E). The CCR5-positive fraction in the PBMC recovered was 49.7%, while that in PHA-PBMC was 27.3%. The mean fluorescence intensity of the CCR5⁺ cell population was 141, compared to the CCR5⁺ cell population in PHA-PBMC with a mean fluorescence intensity of 51. The estimated number of CCR5 expressed on the PBMC recovered on day 3 was 25,348 (as antibody binding sites per cell) while that on PHA-PBMC on day 3 in culture was 8,981 antibody binding sites as examined by quantitative FACS assay. These data indicate that the transplanted human PBMC were intensely activated and rapidly proliferating and expressed high levels of CCR5 on their cell surfaces.

Potent activity of AK602 against R5 HIV-1 *in vitro*. Among SDP derivatives we designed and synthesized, AK602 was identified to be highly potent against a broad spectrum of R5 HIV-1 strains, including MDR clinical R5 HIV-1 isolates *in vitro* with 50% inhibitory concentration (IC_{50}) values of 0.3 to 0.6 nM, although two previously published CCR5 antagonists (TAK779 and SCH-C) were substantially less potent than AK602 (Table 1). AK602 and other CCR5 antagonists failed to inhibit the replication of an X4 HIV-1 strain, HIV-1_{NIJ.3.3}.

Pharmacokinetics of AK602 in hu-PBMC-NOG mice. We examined the pharmacokinetics of AK602 in hu-PBMC-NOG mice by intraperitoneally administering the compound at a dose of 60 mg/kg. Plasma samples were collected periodically up to 12 h and subjected to HPLC analysis. As shown in Fig. 4A, the concentration of AK602 reached the maximal concentration immediately after intraperitoneal administration and decreased rapidly. The calculated plasma half-life in the α -phase of the concentration curve was as short as 29 min.

AK602 persists on cell surface CCR5. As shown above, the plasma half-life of AK602 turned out to be short; however, considering that AK602 possesses such a high affinity to CCR5 and potent activity against R5 HIV-1 *in vitro*, it was thought possible that AK602 would remain attached on cellular CCR5 for an extensive period of time and exert anti-R5 HIV-1 activity even when the compound was depleted from circulation. To examine this possibility, we used two monoclonal antibodies, 45531 and 3A9. When human PBMC were recovered from a hu-PBMC-NOG mouse 2 and 6 h after AK602 administration (60 mg/kg) and stained with 45531, AK602 proved to block the binding of 45531 to CCR5 (Fig. 4B), while AK602 failed to block 3A9 binding to CCR5 (Fig. 4C), suggesting that AK602 did not elicit CCR5 internalization or shedding at all at least for 6 h. We subsequently examined whether AK602 remained on cellular CCR5 with the 45531 monoclonal antibody. When the cells were recovered from mice 2, 6, and 14 h after the AK602 administration, the mean values of the percentage of AK602 occupancy were 85 (four mice), 54 (three mice), and 16 (three mice), respectively. It was calculated that it took about 9 h for AK602 occupancy to be reduced by 50% (Fig. 4D).

Anti-R5 HIV-1 activity of AK602 persistently seen after its removal from culture medium. In another depletion experiment, we exposed CCR5⁺ MAGI cells to AK602 for 30 min, depleted the compound from the culture by thorough washing, incubated the cells for various lengths of time, exposed the cells to HIV-1_{Ba.1}, further cultured the cells for 48 h, and determined whether HIV-1_{Ba.1} infection was blocked by AK602 exposure (Fig. 4E). When the CCR5⁺ MAGI cells were exposed to 0.1 and 1 μ M AK602 and exposed to HIV-1_{Ba.1} immediately afterward, the values for protection were 68 and 85%, respectively. When the cells were exposed to HIV-1_{Ba.1} 4 h after depletion, 49 and 72% of the cells were protected by 0.1 and 1 μ M AK602. When the cells were exposed to HIV-1_{Ba.1} 12 and 24 h after depletion, 57 and 45% of the cells were seen protected by 1 μ M, respectively (Fig. 4E).

Effects of AK602 on CD4⁺ and CD8⁺ cell counts in R5 HIV-1-infected hu-PBMC-NOG mice. PBMC were recovered from murine blood samples collected on days 5, 9, and 16 after R5 HIV-1 inoculation and subjected to flow cytometric analysis for determination of CD4⁺/CD8⁺ cell ratios. As shown in Fig. 5A, in PBMC recovered on day 16 from a representative

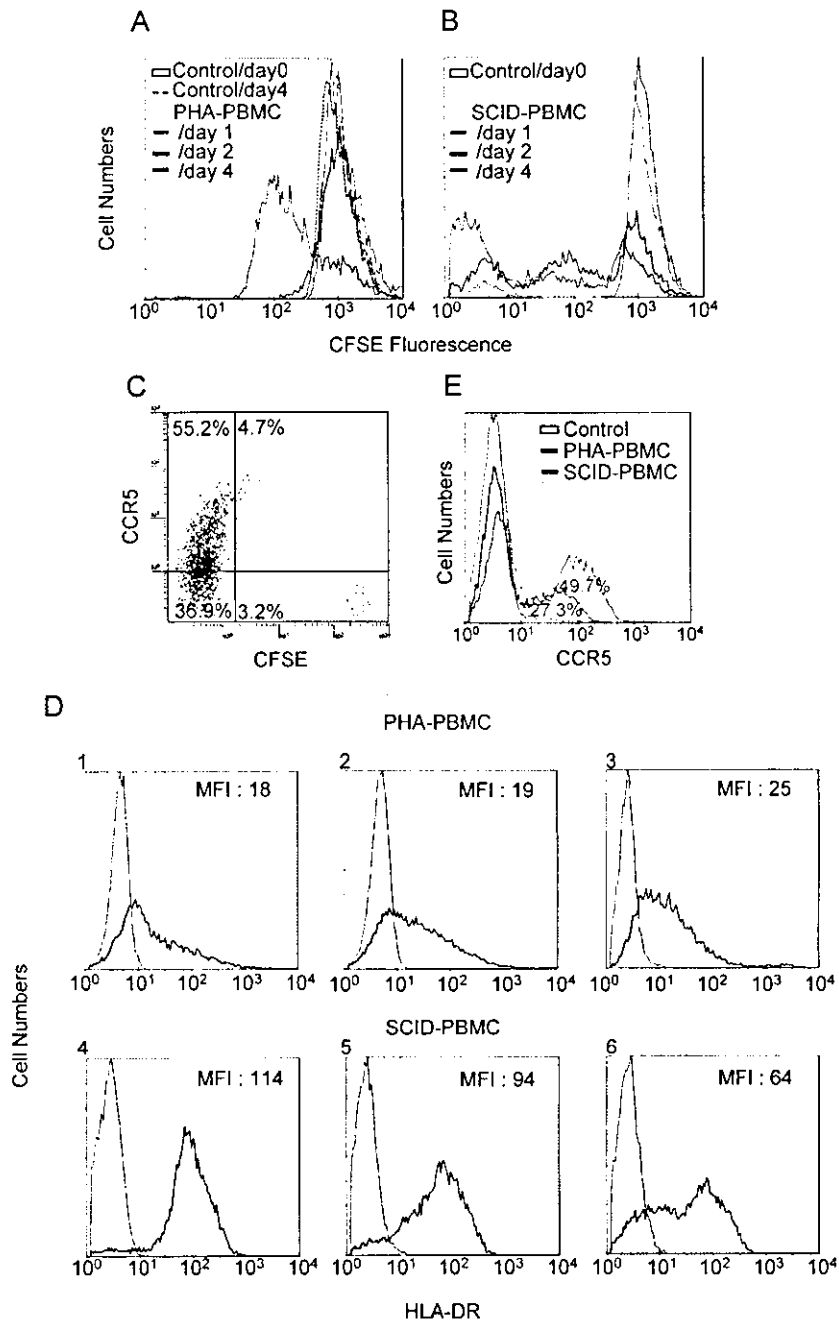


FIG. 3. Transplanted PBMC are intensely activated and express high levels of CCR5. (A and B) Proliferation profiles of PHA-PBMC and transplanted and recovered PBMC. Freshly prepared PBMC were incubated with the vital dye CFSE, and one part of such PBMC preparation was stimulated with PHA, while the other part was intraperitoneally transplanted to mice. On days 1, 2, and 4, the cells were harvested and the fluorescence intensity of CFSE was determined. Note that transplanted PBMC recovered on day 2 had undergone ~4 cycles of proliferation; by day 4, a majority of cells had undergone ~10 cycles and more of proliferation. (C) CCR5 expression level and CFSE intensity in human PBMC harvested from a spleen of hu-PBMC-NOG mouse on day 4. (D) Intense activation of PBMC after transplantation. PBMC stimulated with PHA and cultured for 4 days (panels 1 to 3) and transplanted PBMC recovered from the uninfected mice on day 4 (panels 4 to 6) were stained with an anti-HLA-DR monoclonal antibody. Note that HLA-DR expression levels in transplanted PBMC were much higher than those in PHA-PBMC. (E) CCR5 expression profiles of PHA-PBMC and transplanted PBMC. PBMC stimulated with PHA and cultured for 3 days and transplanted PBMC recovered from the uninfected mice on day 3 were stained with PE-conjugated anti-CCR5 monoclonal antibody 3A9 and subjected to flow cytometric analysis. SCID-PBMC, PBMC transplanted and recovered.

R5 HIV-1-infected, saline-treated mouse, there were only few CD4⁺ cells (3.9% [1.4% + 2.5%]) resulting in a CD4⁺/CD8⁺ cell ratio of 0.05. However, a distinct CD4⁺ cell population (55.1% [4.4% + 50.7%]) resulting in a CD4⁺/CD8⁺ ratio of

1.84 (Fig. 5B) was seen in PBMC recovered from an AK602-treated mouse, and the size of this CD4⁺ cell population was comparable to that seen in a ddi-treated mouse (53.2% [3.8% + 49.4%]) and that in an uninfected mouse (48.9% [3.8% +

TABLE 1. Anti HIV-1 activity of novel SDP derivatives in PBMC^a

Compound	IC ₅₀ value in p24 assay (nM)					
	HIV-1 _{Ba-L} (R5)	HIV-1 _{JRF1} (R5)	HIV-1 _{MOKW} (R5)	HIV-1 _{MM} (R5 _{MDR})	HIV-1 _{JST} (R5 _{MDR})	HIV-1 _{NI4-3} (X4)
AK602	0.5 ± 0.3	0.2 ± 0.1	0.3 ± 0.2	0.7 ± 0.3	0.4 ± 0.2	>1,000
TAK779	14 ± 5	6 ± 2	9 ± 3	12 ± 4	10 ± 3	>1,000
SCH-C	3 ± 2	2 ± 1	2 ± 1.5	2.5 ± 1	2 ± 1	>1,000
ZDV	13 ± 5	7 ± 3	10 ± 6	520 ± 75	64 ± 13	9 ± 5
SQV	8 ± 3	6 ± 2	6 ± 3	212 ± 56	276 ± 44	10 ± 4

^a IC₅₀s were determined by using PHA-PBMC isolated from three different donors, and the inhibition of p24 Gag protein production was used as an endpoint. All assays were conducted in triplicate. The results shown represent arithmetic means (±1 standard deviation) of three independently conducted assays. HIV-1_{MOKW} was isolated from a drug-naïve AIDS patient, and HIV-1_{JST} and HIV-1_{MM} were isolated from patients who received antiretroviral therapy for a long period of time and whose virus loads showed a number of RT and PR mutations. Two previously published CCR5 inhibitors, TAK779 and SCH-C, and zidovudine (ZDV) and saquinavar (SQV) were used as reference compounds.

45.1%), resulting in the ratios of 1.43 and 1.40 (Fig. 5C and D), respectively. Figure 6A illustrates the overall profiles of CD4⁺/CD8⁺ cells ratios on day 16 in the four groups. The mean CD4⁺/CD8⁺ cell ratio in mice (*n* = 7) given saline was 0.1 (range, 0.06 to 0.20). In contrast, the ratios in AK602-

treated mice (*n* = 8) were significantly higher with a mean value of 0.92 (range, 0.23 to 1.89; *P* = 0.001), which was comparable to that in ddI-treated mice (*n* = 9; mean, 1.29; range, 0.38 to 2.68; *P* = 0.001) and uninfected mice (*n* = 7; mean, 1.0; range, 0.50 to 1.49). The numbers of CD4⁺ cells/μl

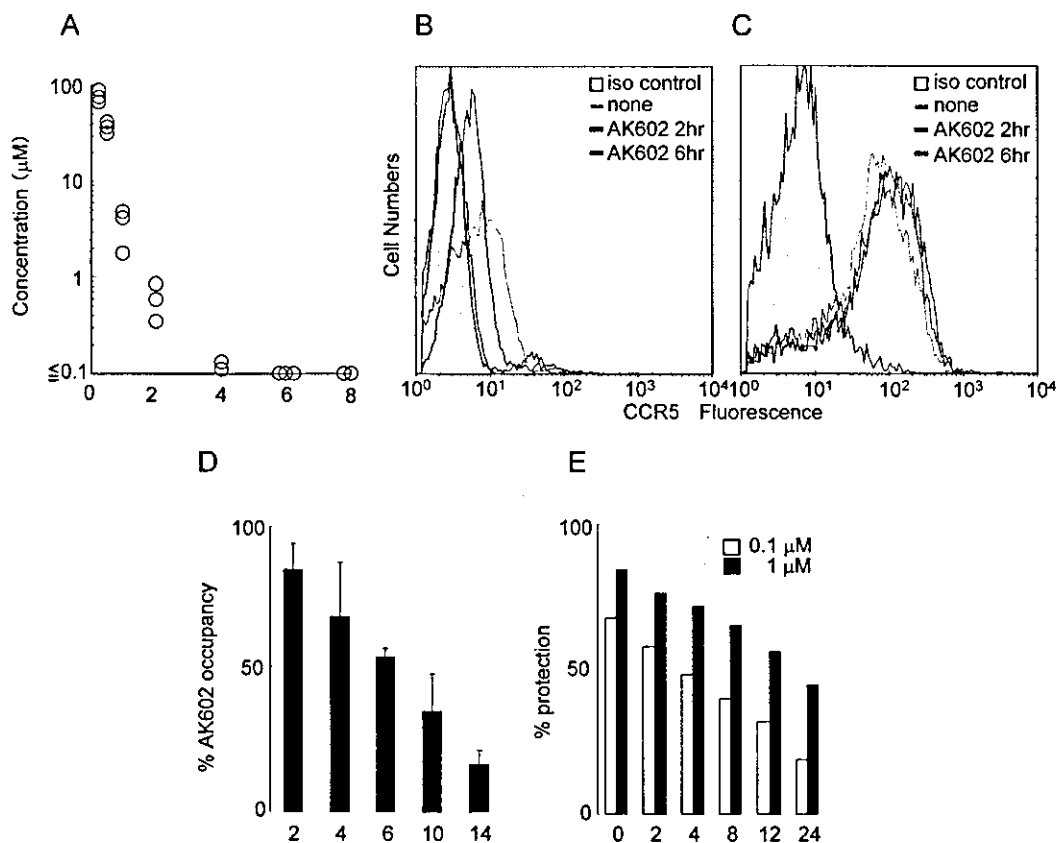


FIG. 4. Pharmacokinetics and persistence of anti-HIV-1 activity of AK602. (A) Pharmacokinetics of AK602. Each mouse was administered AK602 at a dose of 60 mg/kg, and blood samples were taken at 15, 30, 60, 120, 240, 480, and 720 min. Plasma concentrations of AK602 determined by HPLC analysis at 15, 30, 60, 120, and 240 min were 76.2, 36.1, 3.5, 0.6, and 0.13 μM, respectively. AK602 was not detected at later time points. (B and C) No CCR5 internalization or shedding was caused by AK602. Human PBMC were recovered 2 and 6 h after AK602 administration and stained with 45531 (B) or 3A9 (C). (D) Sustained AK602 occupancy on cell surfaces. At indicated periods of time after a bolus of AK-602 (60 mg/kg) was administered to hu-PBMC-NOG mice, PBMC were recovered and the percentages of AK602 occupancy on cellular CCR5 were determined with fluorescein isothiocyanate-conjugated monoclonal antibody 45531. (E) Persistence of in vitro activity of AK602 against R5 HIV-1 after AK602 depletion. CCR5⁺ MAGI cells were exposed to 0.1 or 1 μM AK602 for 30 min and thoroughly washed to deplete AK602 from the medium. The cells were subsequently cultured for the indicated periods of time, exposed to HIV-1_{Ba-L}, and further cultured for 48 h, when the cells were harvested and lysed with Triton X-100-containing PBS. A solution containing chlorophenol red-β-D-galactopyranoside was added, the optical density was measured, and the percentage of protection was determined.

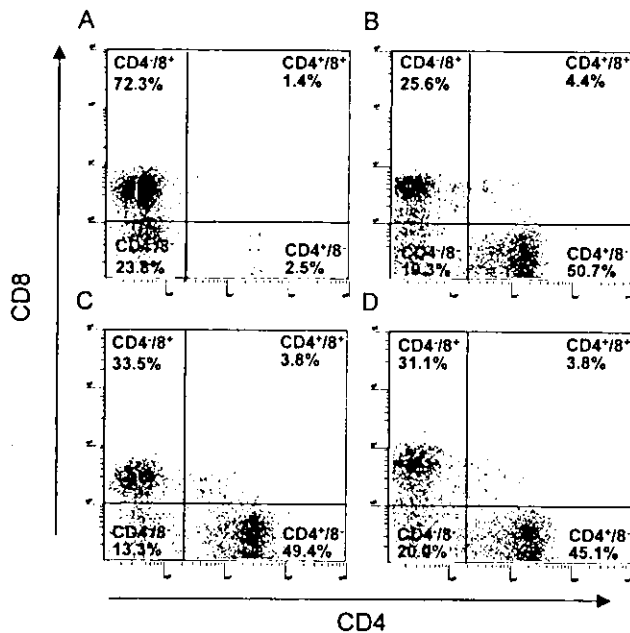


FIG. 5. Effects of AK602 on CD4⁺ and CD8⁺ cell counts in infected hu-PBMC-NOG mice. PBMC recovered on day 16 after R5 HIV-1 inoculation were subjected to flow cytometry. Shown are representative flow cytometric analysis profiles. Note that only 3.9% of CD4⁺ cells were seen (A), resulting in a CD4⁺/CD8⁺ cell ratio of 0.05 in a mouse given saline, while distinct numbers of CD4⁺ cells (55.1 and 53.2%) (B and C) were seen in AK602- and ddI-administered infected mice, resulting in CD4⁺/CD8⁺ cell ratios of 1.84 and 1.43, respectively. In an uninfected mouse (D), 48.9% of cells were positive for CD4, with a CD4⁺/CD8⁺ cell ratio of 1.40.

in saline-treated mice were significantly less than those of AK602-treated, ddI-treated, or uninfected mice (Fig. 6B).

Effects of AK602 on R5 HIV-1 proviral DNA copy numbers and serum p24 levels in R5 HIV-1-infected hu-PBMC-NOG mice. We next asked which population harbored proviral DNA in the cells recovered from R5 HIV-1-infected hu-PBMC-NOG mice, by purifying CD4⁺ and CD4⁻ cell populations and determining proviral DNA copy numbers in each population. As shown in Table 2, more than 99% of proviral DNA was found in CD4⁺ cells and <0.3% of proviral DNA was detected in CD4⁻ cells derived from saline-treated mice, indicating that R5 HIV-1 infection occurred in CD4⁺ cells in the hu-PBMC-transplanted NOG environment. As illustrated in Fig. 6C, the mean number of R5 HIV-1 proviral DNA copies was 2.0×10^5 (range, 2.6×10^4 to 1.7×10^6) per 10^5 CD4⁺ cells in R5 HIV-1-infected mice ($n = 7$) given saline. However, values for mice in groups given AK602 and ddI were 1.3×10^3 (range, 2.3×10^2 to 7.9×10^3 ; $P = 0.001$) and 1.8×10^2 (range, $<10^2$ to 7.9×10^2 ; $P = 0.001$), respectively.

The amounts of R5 HIV-1 p24 in serum were also found to be very high in saline-treated mice, with a mean amount of 1.1×10^5 pg/ml (range, 3.1×10^4 to 2.8×10^5 pg/ml). AK602 and ddI were found to significantly suppress the serum p24 amounts as examined on day 16 with a mean amount of 5.6×10^3 pg/ml (range, 8.1×10^2 to 2.1×10^4 pg/ml; $P = 0.001$) and 7.1×10^2 pg/ml (range, 1.3×10^2 to 1.1×10^4 pg/ml; $P = 0.001$), respectively (Fig. 6D).

AK602 suppressed R5 HIV-1 viremia in hu-PBMC-NOG mice. As described above, the PBMC transplanted to NOG mice were intensely activated in the xenogenic environment and had undergone ~ 4 cycles of proliferation by day 2; a majority of the cells had undergone ≥ 10 cycles of proliferation by day 4 (Fig. 3B). These data suggested that R5 HIV-1 might extensively replicate in the hu-PBMC-NOG mice immediately after R5 HIV-1 inoculation. When we collected blood samples on days 5, 9, and 16 following the inoculation and determined R5 HIV-1 RNA copy numbers in infected, saline-treated mice ($n = 7$), the geometric mean copy number was 8.6×10^3 /ml (range, 1.7×10^3 to 1.0×10^5) on day 5 and rapidly increased to 1.9×10^5 /ml (range, 2.2×10^4 to 3.0×10^6) on day 9; by day 16, the mean copy number had reached 7.7×10^5 /ml (range, 2.6×10^5 to 3.0×10^6 /ml). However, AK602 significantly suppressed viremia by ~ 1.1 log, as examined on day 5; the mean numbers of R5 HIV-1 RNA copies in AK602-administered mice were 1.6 and 1.8 logs lower than those in saline-treated mice examined on days 9 and 16, respectively (Fig. 7). Comparable viremia suppression was seen in the mice receiving ddI (Fig. 7). It was noted that although AK602 did not completely prevent the viremia from further increasing after day 5, there was a clear reduction in the viremia increase rates. The mean slopes (change in RNA copies per day over the range of data from 5 to 16 days) for the group receiving saline was 0.167 ± 0.042 , whereas those for the AK602 and ddI groups were 0.102 ± 0.041 and 0.091 ± 0.037 , respectively. Thus, the rates of increase in the AK602 ($P = 0.0057$) and ddI ($P = 0.0023$) mice were significantly lower than that for the mice given saline, indicating that both of the agents significantly inhibited R5 HIV-1 replication in this mouse model over the range of days evaluated. No apparent AK602- or ddI-associated adverse effects were seen throughout the study period.

DISCUSSION

In the present hu-PBMC-NOG mouse model, human CD4⁺/CD8⁺ cell ratios went down to 0.1 by 16 days after R5 HIV-1 inoculation, the amounts of proviral DNA and p24 gag antigen reached 10^5 to 10^6 copies/ 10^5 CD4⁺ cells and 10^5 pg/ml, respectively (Fig. 6), and no mice failed to be infected with R5 HIV-1. It is noteworthy that the use of NOG mice provides a higher engraftment rate than with other SCID mice such as NOD/Shi-SCID mice treated with anti-NK cell antibody or the β_2 -microglobulin-deficient NOD-SCID mice (10). With NOG mice, the chimeric rate of 30 to 40% is achieved, and cord blood CD34⁺ cells have been shown to "take" with as few as 100 cells (10). Moreover, all infected mice developed high levels of R5 HIV-1 viremia by day 16, reaching as high as 10^6 copies/ml (Fig. 7). It is worth noting that the notably high levels of HIV-1 viremia seen in the present mouse model by 16 days after R5 HIV-1 exposure can be seen only on acute infection or up to 10 years after HIV infection in humans (3, 4).

In the present study, we found that the conspicuous susceptibility to the infectivity and replication of R5 HIV-1 in these mice appeared to stem from the hyperactivation of the implanted human PBMC. The implanted PBMC were highly activated in the xenogenic environment, expressed quite high

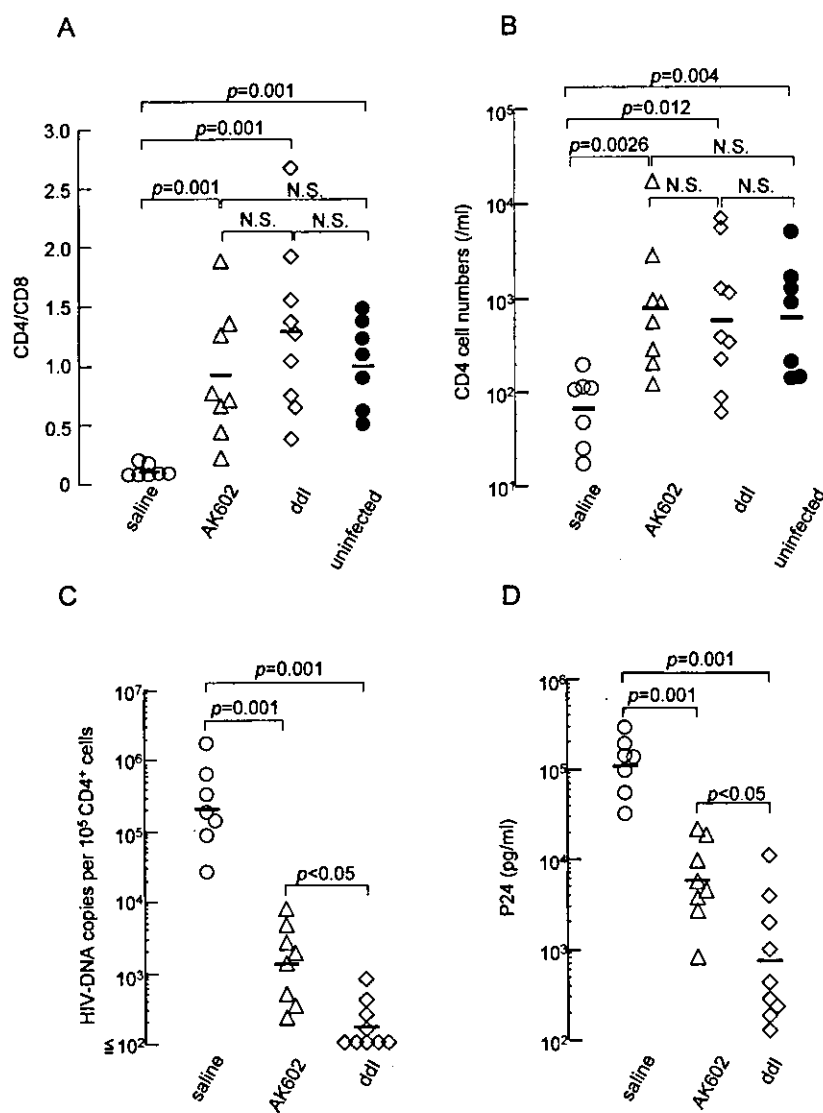


FIG. 6. Effects of AK602 on CD4⁺/CD8⁺ ratios and the amounts of proviral DNA and HIV-1 p24 in infected hu-PBMC-NOG mice. (A) Overall profiles of CD4⁺/CD8⁺ cell ratios. Note that the mean CD4⁺/CD8⁺ cell ratio in mice given saline (*n* = 7) was 0.1, while those in mice given AK602 or ddI were 0.92 and 1.29, respectively. The mean ratio in uninfected mice was 1.0. (B) Numbers of CD4⁺ cells per microliter in each mouse group. (C) HIV-1 proviral DNA copy numbers in CD4⁺ cells from each mouse group were determined by real-time PCR assay. Values are shown per 10⁵ CD4⁺ cells, as described in Materials and Methods. Note that the mean number of HIV-1 proviral DNA copies was 2.0 × 10⁵ per 10⁵ CD4⁺ cells in mice given saline, while those in AK602- and ddI-treated groups were 1.3 × 10³ and 1.8 × 10² per 10⁵ CD4⁺ cells (both, *P* = 0.001), respectively. (D) Amounts of plasma p24 antigen. Note that the amounts of p24 in plasma were high in saline-treated mice while AK602 and ddI significantly suppressed the serum p24 amounts as examined on day 16 after HIV-1_{Ba-L} inoculation. The short bars indicate the arithmetic (A) and geometric (B, C, and D) means obtained.

levels of HLA-DR, and rapidly and continuously proliferated immediately after intraperitoneal infusion (Fig. 3A, B, and D). Moreover, the implanted PBMC expressed as much as 2.8-fold-higher levels of CCR5 on day 3 following implantation compared to PHA-PBMC on day 3 in culture (Fig. 3E). The combination of rapid proliferation and high levels of CCR5 expression of the implanted PBMC should explain the reason R5 HIV-1 rapidly replicated in the hu-PBMC-NOG mice and presented such high levels of R5 HIV-1 viremia. In this regard, only a few groups to date have documented the levels of viremia in the scientific literature. Among them are those by Garaci et al. (8) and Koyanagi et al. (14). The former documented

high levels of viremia with a peak of 2.67 × 10⁶ copies/ml in hu-PBL-NOD-SCID mice in which HIV-1-infected macrophages were inoculated, unlike our NOG mouse model where HIV-1 was directly inoculated. The latter report by Koyanagi et al. does not have viremia data but has data on p24 levels with a geometric mean of 11,092 pg/ml on day 14 after HIV-1 inoculation. However, the variation was much greater (178 to 1,434,444 pg/ml). Thus, one can say that the present model provides a greater reproducibility of high viremia levels than the mouse system reported by Koyanagi (14). It should be noted that the high levels of viremia and high engraftment rate achieved in this mouse model made it possible to monitor the

TABLE 2. Comparison of HIV-1 proviral DNA in human CD4⁺ and CD4⁻ cell fractions^a

Sample	HIV-1 DNA copies (10 ⁵ cells)		
	SCID-PBMC	CD4 ⁺ cells	CD4 ⁻ cells
Saline 1	138,858	162,193	461
Saline 2	135,967	117,949	<100
Saline 3	83,863	94,590	<100
AK602 1	3,390	2,300	<100
AK602 2	5,575	4,606	<100
AK602 3	1,925	1,398	<100
ddI 1	301	516	<100
ddI 2	793	1,317	<100
ddI 3	<100	118	<100

^a HIV-1 proviral DNA copy numbers were determined by real-time PCR assay of unseparated human PBMC and purified CD4⁺ and CD4⁻ cells, following recovery from hu-PBMC-NOG mice. Values are shown per 10⁵ cells, as described in Materials and Methods.

changes in the viremia levels periodically in the same set of mice without sacrificing them, while most of the previously described SCID mouse models required mice to be sacrificed at each time point of testing (25, 29, 30) or needed further in vitro coculture of the PBMC recovered from the mice with freshly prepared uninfected target cells for an additional period of days (9, 34).

We demonstrated in this study that a novel SDP derivative, AK602, exerted highly potent activity against laboratory and primary R5 HIV-1 strains as well as MDR R5 HIV-1 variant with IC₅₀ values of subnanomolar concentrations (Table 1). It should be noted that AK602 represents a novel SDP derivative, which binds to human CCR5 but not to human CXCR4, CCR1, CCR2, CCR3, CCR4 or murine CCR5; blocks the binding of MIP-1 α to CCR5 with an extremely high affinity (K_d values of ~3 nM); potentially blocks HIV-1-gp120/CCR5 binding; and exerts potent activity against a wide spectrum of laboratory and primary R5 HIV-1 isolates including MDR HIV-1 and HIV-1 strains of various clades with IC₅₀ values of 0.2 to 0.6 nM in vitro (K. Maeda, H. Ogata, S. Harada, Y. Tojo, T. Miyakawa, H. Nakata, Y. Takaoka, S. Shibayama, D. Fukushima, J. Moravek, E. Arnold, and H. Mitsuya, 11th Conf. Retrovir. Opp. Infect., abstr. 540, 2004; J. Demarest et al., XV Int. AIDS Conf., abstr. WeOrA1231, 2004). The plasma half-life of AK602 in the hu-PBMC-NOG mice, however, proved to be as short as 29 min when the agent was administered intraperitoneally (Fig. 4A). Considering that AK602 possesses such a high binding affinity to CCR5, we presumed that AK602 could remain on CCR5 for an extended period of time even after the agent was removed from the bloodstream in mice. The high and extensive level of AK602 occupancy observed in PBMC recovered from mice receiving AK602 substantiated this presumption (Fig. 4D). The subsequent in vitro experiment in which CCR5⁺ MAG1 cells were incubated with AK602 but exposed to R5 HIV-1 after the removal of the compound from the culture medium showed that AK602's anti-R5 HIV-1 activity can persist for an extensive period of time even if AK602 is no longer present in the culture (Fig. 4E). It is of note that unlike certain reports of in vivo anti-HIV-1 activity of

chemokine antagonists which were administered before HIV-1 inoculation, thus demonstrating prophylactic effects of such agents (9, 30), the present system demonstrates anti-HIV-1 treatment after the establishment of HIV-1 infection, analogous to antiviral therapy in clinical settings.

When highly active antiretroviral therapy exerts its potent antiviral effects in clinical settings, a decrease in HIV-1 viremia is seen often within weeks, ultimately resulting in undetectable viremia; however in the present study, the viremia levels in mice receiving AK602 or ddI continued to increase although the rate of increment significantly declined (Fig. 7). The failure of AK602 and ddI to decrease viremia levels could be due in part to such a rapid viral replication in hyperactivated and proliferating CD4⁺ cells. As discussed earlier, PBMC recovered from the hu-PBMC-NOG mice were highly positive for CCR5 and HLA-DR (Fig. 3D and E), compared to the levels of activation seen in the same donor's PHA-PBMC. It should be noted, however, that the mean numbers of proviral DNA copies on day 16 in mice receiving AK602 and ddI were 1.3×10^3 and 1.8×10^2 per 10⁵ CD4⁺ cells, respectively (Fig. 6C), suggesting that most CD4⁺ cells (98.7 and 99.8% on average, respectively) were free of HIV-1 and proliferating in those

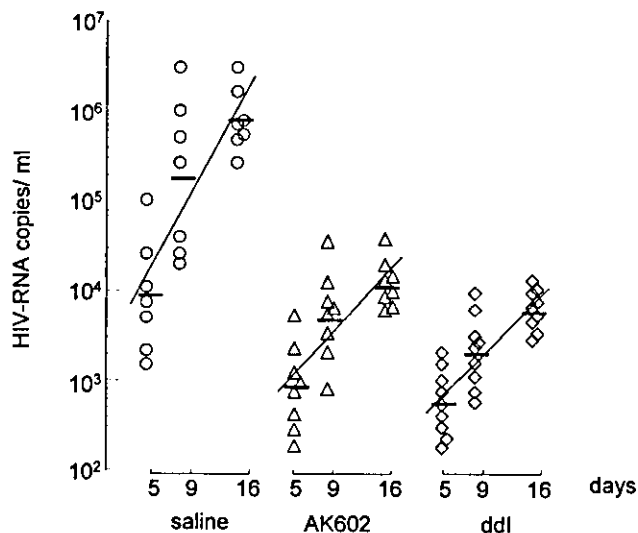


FIG. 7. AK602 suppresses R5 HIV-1 viremia in hu-PBMC-NOG mice. Blood samples were collected on days 5, 9, and 16 after inoculation and were subjected to the determination of R5 HIV-1 RNA copy numbers. Note that the copy numbers in saline-treated mice rapidly increased and reached $\sim 10^6$ /ml by day 16, while AK602 significantly suppressed the viremia by 1.6 and 1.8 logs as examined on day 9 ($P = 0.001$ compared to saline-treated mice) and day 16 ($P = 0.001$), respectively. Comparable viremia suppression was seen in ddI-treated mice, except on day 16, when ddI activity was greater than that of AK602 ($P = 0.027$). Note that there was a clear reduction in the rate of increase of viremia as well. When the values of log₁₀ HIV-1 RNA copies were calculated and the slopes corresponding to the rates of increase per day were determined, the resulting mean slope (solid line) for the saline-treated mice was 0.167 ± 0.042 , whereas those for the AK602- and ddI-treated mice were 0.102 ± 0.041 and 0.091 ± 0.037 , respectively. The increase rate for saline-treated mice was significantly higher than those of AK602-treated mice ($P = 0.0057$) and ddI-treated mice ($P = 0.0023$), respectively. The horizontal bars and solid lines represent the geometric means of HIV-1 RNA copy numbers and the slopes calculated, respectively.

mice on day 16 after the virus inoculation, if one copy of proviral DNA was postulated to reside in one CD4⁺ cell.

One of us (Y.K.) previously attempted to investigate the mechanism of CD4⁺ cell depletion seen in individuals with HIV-1 infection by employing a PBMC-transplanted NOD (NOD/Shi) *scid/scid* mouse system (24). Massive apoptosis was observed in HIV-1-uninfected CD4⁺ cells in the spleens of the HIV-1-infected NOD-*scid/scid* mice. A combination of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling and immunostaining for death-inducing tumor necrosis factor (TNF) family molecules showed that apoptotic cells were frequently found in conjugation with TNF-related apoptosis-inducing ligand (TRAIL)-expressing CD3⁺ CD4⁺ human T cells. Further observation that a neutralizing anti-TRAIL antibody inhibited the development of CD4⁺ cell apoptosis suggested that a large number of HIV-1-uninfected CD4⁺ cells undergo TRAIL-mediated apoptosis, contributing to the marked depletion of CD4⁺ cells (24). The observation by Miura and his colleagues that the number of TRAIL-positive cells was consistently higher in HIV-1-infected mice than in uninfected ones makes it apparent that TRAIL expression is induced upon HIV-1 infection (23, 24). In this regard, the present observation that AK602 and ddI potently blocked the decrease in CD4⁺ cells in spite of the rather increasing HIV-1 viremia in the face of AK602 or ddI (Fig. 7) suggests that the mere presence of viremia might not be sufficient for the HIV-induced apoptosis in CD4⁺ cells. Our observation that most surviving CD4⁺ cells in mice receiving AK602 or ddI were free of HIV-1 (see above) suggests that these anti-HIV-1 agents might block not only de novo HIV-1 infection, but also bystander killing of uninfected CD4⁺ cells. The present data also suggest that a certain factor(s) such as cytokines produced by the freshly HIV-1-infected cells might mediate the apoptosis of bystander CD4⁺ cells through the upregulation of TRAIL expression, death receptors (e.g., DR4 and DR5), and/or downregulation of decoy receptors (e.g., DcR1 and DcR2) (26, 27). However, experiments with a combination of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling and TNF family molecules have to be conducted for better understanding of the bystander killing in regard to AK602's effects.

It is of note that several CCR5 antagonists are currently in various stages of development. AK602 has recently been administered to healthy adult subjects in a phase I clinical trial and shown to bind to CCR5 for an extended period of time, suggesting that an oral formulation with fewer administrations and lower dosage is possible for AK602 as a therapeutic agent for HIV-1 infection (J. Demarest, K. Adkison, S. Sparks, A. Shachoy-Clark, K. Schell, S. Reddy, L. Fang, K. O'Mara, S. Shibayama, and S. Piscitelli, 11th Conf. Retrovir. Opp. Infect., abstr. 139, 2004). Taken together, our observations that plasma viral load reached $\sim 10^6$ RNA copies/ml and that AK602 potently inhibited the replication of R5 HIV-1 strongly suggest that the present hu-PBMC-NOG mouse AIDS model could serve as a useful instrument for analyzing the pathogenesis of HIV-1 infection and testing the efficacy of antiviral agents.

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