TABLE 2. Ex vivo neutralizing activity of KD-247 against HIV-1 present in PBMC cultures established using cells from HIV-infected individuals[a]

Patient	HIV-1 Env V3 sequence (no. of clones)	PBMCs, (no. of cells/well)	KD-247 (μg/ml)	p24 (log_{10} pg/ml)
KU008	CTRPHNNTRKSIHIGPGRAFYATGDIIGNIRQAHC (3)	6.5×10^5	0	3.93
	----------E---D--R-- (2)		60	0.37
	----------E---D----- (1)		240	0.08
	----------D----- (1)			
KU045	CTRPNNNTRKGIHIGPGRAFYGTDIVGDIRQAHC (5)	7.3×10^5	0	3.70
	----------E-T-N----Y- (2)		60	0.88
	----------N------ (1)		240	0.56
KU037	CTRPNNNTRKSIPIGPGRAFYATGDIIGDIRKAHC (3)	1.3×10^6	0	3.81
	-------I---------- (1)		60	3.86
	-I------G---------- (1)		240	0.25
KU040	CTRPNNNTRKSVHIGPGRAWYATGEIIGNIRQAHC (2)	8.0×10^5	0	4.12
	----------A----F---------- (1)		60	2.34
	----------I--------H---------- (1)		240	2.62
	---H-------I-L---G--H---D--------- (1)			

[a] Ex vivo neutralization activity was directly detected by using $CD8^+$ cell-depleted PBMCs from HIV-infected individuals as described in Materials and Methods.
[b] The number of analyzed DNA clones from each patient is indicated in parentheses. Dashes indicate sequences identical to those of the upper major clone from each patient.

ation of viruses from PBMCs of KU008 was reduced in a dose-dependent manner, with 3.56- and 3.85-log reductions in the culture supernatants, respectively; reductions of 2.82 and 3.14 logs of virus generation from PBMCs of KU045 were also detected in the presence of 60 and 240 μg/ml of KD-247, respectively, KU037 showed a reduction of 3.56 logs at only 240 μg/ml. However, KU040 showed no dose-dependent suppressive effects of virus generation by KD-247. When the irrelevant antibodies of Cβ1 and normal serum IgG were added to cell cultures, they showed no suppressive effects on virus generation (data not shown). These results demonstrate that KD-247 effectively neutralizes nonpassage viruses generated in the primary culture of PBMCs from individuals infected with HIV-1 clade B with neutralization sequence motifs matching that of the quasispecies, IGPGR.

Induction of complete protection of monkeys against a highly pathogenic SHIV strain by a single passive transfer of a high dose of KD-247. PBMCs from 12 juvenile male cynomolgus monkeys were first evaluated in vitro to establish their susceptibility to infection with the SHIV C2/1 challenge stock in standard viral infectivity assays (35, 37) (data not shown). Challenge virus SHIV C2/1 originated from SHIV 89.6 but did share an identical envelope sequence with the parental strain, $HIV\text{-}1_{89.6}$, and showed 17 nucleotide mutations with amino acid changes (1, 34). The neutralization sensitivity of SHIV C2/1 to KD-247 was found to be similar to that of $HIV\text{-}1_{89.6}$, with an IC_{90} and IC_{50} of 5 and 0.5 μg/ml in human PBMC-based neutralization assays, respectively (Table 1, laboratory isolates, clade B and SHIV-B), suggesting that the neutralization potency of KD-247 in vitro might be sufficient to warrant passive transfer experiments.

Of the 12 monkeys, 5 were inoculated with KD-247, 2 were inoculated with control normal human IgG (NHIgG) (45 mg/kg), and the remaining 5 were given saline alone. Of the five animals receiving KD-247, two were given a dose of 45 mg/kg, two received 30 mg/kg, and one received 15 mg/kg. Twenty-four hours after antibody transfer, all 12 monkeys were given an intravenous challenge of 20 $TCID_{50}$s/ml SHIV (Fig. 1). At the time of viral challenge, the plasma concentrations of KD-247 were 151, 443, 496, 866, and 678 μg/ml of the antibody in immune sera from monkeys 3968, 3969, 3972, 4092, and 4099, respectively (Fig. 1a). The area under the plasma concentration time curve (AUC) values for monkeys 3968, 3969, 3972, 4092, and 4099 were calculated from the antibody concentration data to be 1.8, 3.5, 5.0, 6.5, and 5.6 mg · day/ml, respectively.

The percentage of $CD4^+$ T cells and the levels of plasma viremia were also monitored after SHIV challenge (Fig. 1b and c). All monkeys that were intravenously inoculated with normal human IgG or saline showed a loss of $CD4^+$ T cells within 7 days of viral challenge, accompanied by plasma viremia reaching 10^7 to 10^8 viral RNA copies/ml (data from the five control monkeys that received saline alone are not shown). Of the two control monkeys that received 45 mg/kg of NHIgG, both seroconverted against SHIV p27 antigen (monkeys 3967 and 3974) (Fig. 1d). At autopsy, all control monkeys showed $CD4^+$-T-cell depletion in lymphoid organs, a finding consistent with our previous observations using this model (35, 37).

Both monkeys that received a single high dose of 45 mg of KD-247 per kg of body weight prior to SHIV challenge were completely protected from viral challenge, maintaining stable $CD4^+$-T-cell counts and not seroconverting or exhibiting plasma viremia (Fig. 1b to e, monkeys 4092 and 4099, indicated by red lines and red characters). When evaluated at autopsy using PCR for SHIV *gag* proviral DNA, their tissues showed no sign of infection (data not shown). The titers in plasma resulting from 100% in vitro neutralization against 100 $TCID_{50}$s of the challenge virus at the time of virus challenge were 1:160 in both monkeys 4092 and 4099. The titers in partially protected monkeys 3969 and 3972 were 1:40 and 1:80, respectively. No neutralization activity of less than 1:10 was measured in the animals receiving 45 mg/kg of NHIgG (monkeys 3967 and 3974). Thus, although the highest titers of neutralization activities were detected in plasma from protected animals, the neutralization activity was high even in animals with only partial protection.

Administration of lower doses of KD-247, 30 mg/kg to two monkeys (monkeys 3969 and 3972, indicated by blue lines and

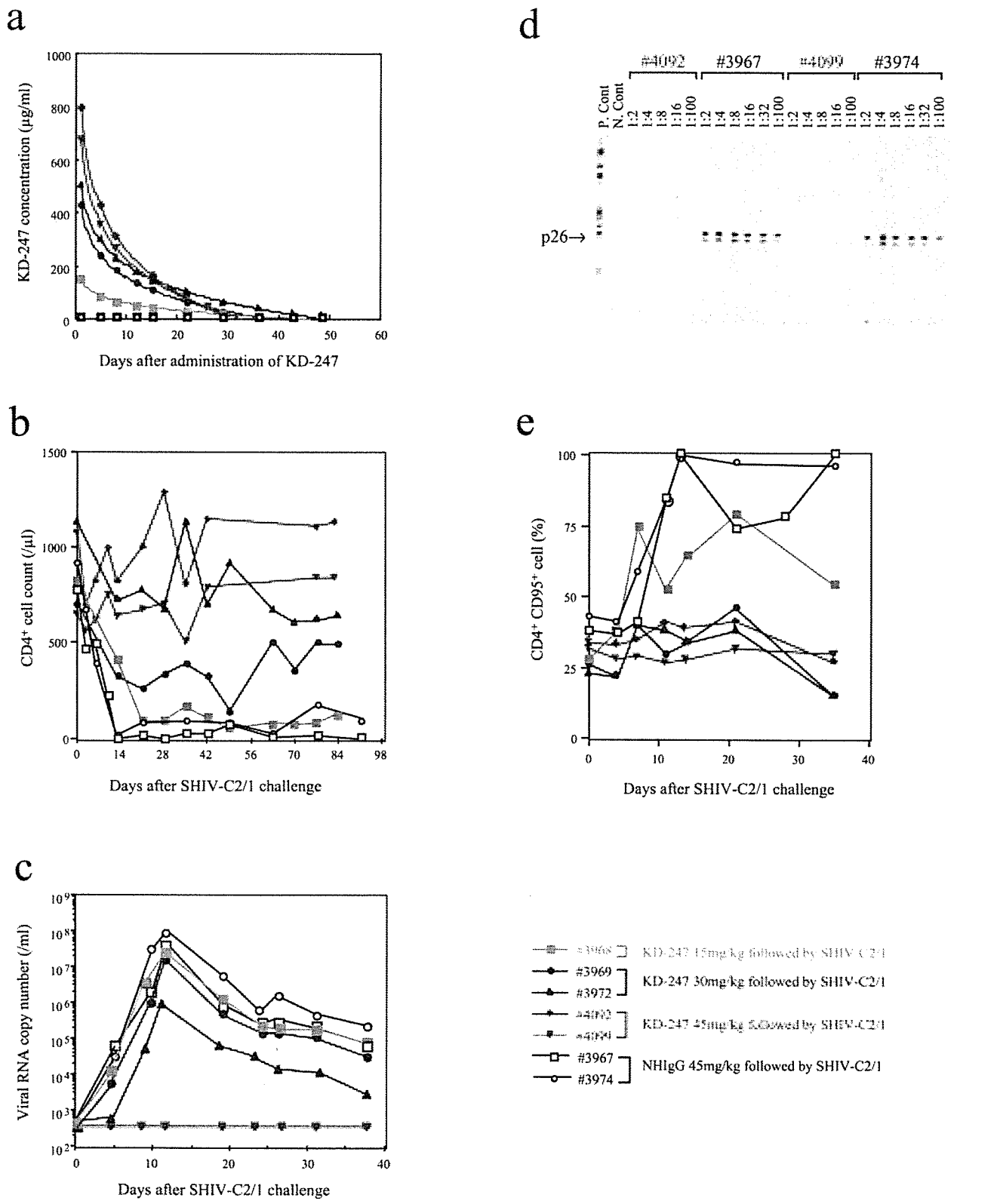

FIG. 1. KD-247 efficiently protects monkeys from pathogenic virus challenge. A total of 12 cynomolgus monkeys were used for virus challenge studies with SHIV C2/1. In the first group, five monkeys were intravenously inoculated with various doses of KD-247, followed by 20 $TCID_{50}$s of SHIV C2/1 challenge 24 h after antibody transfer. Monkeys in the second and third groups were injected prior to virus challenge with either 45 mg/kg of normal human immunoglobulin (two monkeys) or saline alone (five monkeys). The following parameters were measured in monkeys given KD-247: (a) concentration of KD-247 in plasma following passive transfer, (b) $CD4^+$-T-cell counts, (c) plasma viremia, (d) Western blot analysis using an HIV-2 Western blot kit (Diagnostics Pasteur, Marnes-La-Coquette, France) (6) of serum samples obtained at autopsy from monkeys given a single high dose (45 mg/kg) of KD-247 (monkeys 4092 and 4099) or NHIgG controls (monkeys 3967 and 3974), and (e) CD95 antigen expression on PBMCs from monkeys challenged with SHIV.

blue characters in Fig. 1) and 15 mg/kg to one monkey (monkey 3968, indicated by green lines and green characters in Fig. 1), afforded partial protection from SHIV infection. Monkey 3972 (Fig. 1, closed triangle with blue line) showed better partial protection than monkey 3969, which received 30 mg/kg of antibody. That superior degree of partial protection may be related to better blood concentration of the antibody and to better AUC values. All three monkeys described above seroconverted against SHIV p27 antigen (data not shown), but their loss of $CD4^+$ T cells seemed to be inversely proportional to the plasma concentration of KD-247 (Fig. 1a and b). Although the $CD4^+$-T-cell decline indicated minimal protection in the monkey given 15 mg/kg of KD-247 (monkey 3968) (Fig. 1b), CD95 antigen expression, a marker for cell stimulation, was significantly lowered in this animal and completely inhibited in the other four monkeys receiving KD-247 (Fig. 1e), suggesting that KD-247 significantly suppressed PBMC stimulation by the virus challenge in these animals (monkeys 3969, 3972, 4092, and 4099).

These results therefore demonstrate that KD-247 efficiently neutralizes primary HIV isolates regardless of cell tropism. Furthermore, passive immunization with a single dose of 45 mg of antibodies per kg of body weight 24 h prior to viral challenge completely protected animals from viral challenge, showing that at high concentrations, KD-247 lowers the viral load and induces sterilizing immunity in the monkey model.

DISCUSSION

In this study, KD-247 proved an effective antiviral agent for the targeting of phenotype-matched viruses, one capable of both in vitro neutralization of primary isolates and in vivo passive transfer of the antibody as well as of suppressive effects against ex vivo generation of HIV from HIV-infected individuals. Although it has already been established that brief immunizations with a V3 peptide can elicit neutralizing antibodies to homologues of the CXCR4-tropic virus, the limitations of anti-V3 antibodies have been known for over a decade (8, 13, 16, 28). Also, at reasonable IC_{50}s, the anti-V3 antibodies did not neutralize CCR5-tropic strains. In the accompanying paper (8a), we described the derivation of a humanized MAb, KD-247, that was produced by sequential immunization using six different HIV-1 Env V3 peptides derived from HIV-1 clade B field isolates. We suggested that KD-247 could potentially overcome the previous limitations to immunologically exploiting the anti-V3 antibody induced by brief immunization protocols, i.e., its extraordinary sequence variability and the associated isolate specificity of anti-V3 antibodies (27, 38). The findings of our current study suggest that KD-247 may curb the spread of viral infection and reduce viral loads in HIV-infected individuals who have been determined to share the V3 tip sequence of the virus by virus neutralization phenotype-matching analysis.

In vitro, KD-247 has potent neutralizing activity against a variety of primary HIV-1 clade B isolates, including CCR5-tropic viruses, at low concentrations. We found that KD-247 neutralized a variety of clade B primary viruses containing IGPGR V3 sequences, although its neutralization ability was affected by some of the surrounding amino acids of the V3 tip region, as discussed in the accompanying paper (8a). Based upon these results, we should be able to predict the neutralization ability of KD-247 by prior sequencing of the HIV-1 Env V3 region of the target virus. Using the previously published sequences found in the Los Alamos HIV-1 sequence database, we determined that the IGPGRA sequence is present in the majority of HIV-1 clade B isolates (45) to which KD-247 would be expected to have cross-neutralization activity. Moreover, KD-247 significantly curbed the generation of primary HIV-1 quasispecies in ex vivo cultures of $CD8^+$-T-cell-depleted PBMCs from seropositive individuals. However, as described above, the major limitation of KD-247 as an antiviral agent is its inability to neutralize variants expressing amino acid alterations in the binding site PGR motif and additional amino acids.

What are the properties that make KD-247 an effective neutralizer of CCR5-tropic viruses? First, the site-specific binding of KD-247 to epitopes on the virus envelope glycoprotein seems to be key to its virus neutralization ability. Indeed, the results of the Pepscan analysis reported in the accompanying paper suggest that KD-247 can react with core V3 sequences from various HIV-1 clade B isolates (8a). The shortest peptide that was reactive with KD-247 was IGPGR, but that epitope was stabilized by the addition of one or more amino acids. Furthermore, IGPGRA and GPGRAF sequences occur in the majority of HIV-1 isolates from donors in the United States (17). The results of Pepscan with replacement peptides also suggest that KD-247 has broad binding activity to HIV-1. While the number of amino acid substitutions tolerated in the central PGR sequence of the V3 tip peptide was small, replacement of amino acids in the flanking region was relatively permissible. Second, ex vivo neutralization assays using patient-derived isolates containing APGR and GPGG sequences in the V3 tip showed incomplete neutralization (Table 2, KU040). Thus, KD-247 would be expected to bind with HIV-1 quasispecies having a recognition sequence similar to the neutralization phenotype. Third, as the accompanying paper demonstrates, high-affinity antibody binding is apparently required for neutralization, because the kinetic parameters of KD-247 were identified to be fast on and slow off rates, similar to those of a type-specific MAb, Rμ5.5, although the equilibrium dissociation constant value of KD-247 for binding to a control SP1 peptide was higher than that of Rμ5.5 (8a). This is a reasonable assumption, since the epitope of KD-247 (IGPGR) is shorter than that of Rμ5.5 (IHIGPGRAFYT). The high association rate of KD-247 might be responsible for exerting the observed cross-neutralization activity against various primary isolates. These results are consistent with the hypothesis that virus neutralization can be explained by the kinetic parameters of antibody binding.

Most recent passive transfer studies with monoclonal antibodies used common combinations of broadly cross-reactive human MAbs capable of neutralizing primary HIV-1 isolates. In monkeys, human MAbs b12 (29) and 2G12 (20) were shown to induce complete and partial protection, respectively, against viral challenges. In contrast, the MAb chosen for this study, KD-247, is a humanized antibody induced by sequential immunization with a set of V3 peptides from primary isolates. Because the KD-247 IC_{90} value from an in vitro neutralization assay in our study, 5.0 μg/ml of the antibody, approximates that obtained by a single antibody, b12 (3), and a combination of

the two MAbs 2F5 and 2G12 or a triple combination of HIVIG, 2F5, and 2G12, as previously reported (41, 43), we postulated that KD-247 was sufficiently potent to achieve protection of monkeys against a pathogenic SHIV challenge. Since our previous experience (9) has taught us to expect approximately 500 to 1,000 μg/ml in sera from monkeys passively immunized with 30 to 45 mg of antibody per kg of body weight, the potency of KD-247 should prove sufficient for passive transfer experiments of effective antibodies in animals in vivo. We also expected that a single passive transfer of KD-247 via inoculation with 15 and 30 mg of antibody would result in approximately 150 to 500 μg/ml of plasma concentration at the time of viral challenge. As expected, we found an AUC value of 1.8 to 5.0 mg · day/ml. Consequently, we found that animals passively immunized with 45 mg/kg of KD-247 showed 678 and 866 μg/ml of KD-247 in plasma at the time of viral challenge and an AUC value of 5.6 and 6.5 mg · day/ml. Those animals were provided sterile protection against intravenous challenge with the pathogenic virus SHIV C2/1. The protective endpoint titers of neutralization antibodies in plasma at the time of virus inoculation were 1:160 in both animals that elicited sterile immunity, and a high titer of neutralization activity in plasma was similarly detected in completely protected monkeys, as described previously by Nishimura et al. (26) and Parren et al. (29). Thus, the high titers of neutralization activity in plasma confer sterile protection against viral challenge in the passively immunized animals with neutralizing antibodies. Furthermore, the pharmacokinetic information consisting of the plasma concentration of the neutralizing antibodies at the time of viral challenge and the AUC value may be closely related to the ability of the antibody to provide sterile protection against viral challenge. Since those protected macaques demonstrated the inhibition of $CD4^+$ cell loss, the pharmacokinetic properties of KD-247 may also be closely associated with the inhibition of $CD4^+$ cell decline in the peripheral circulation of the challenged monkeys.

In this study, we also detected lower viremia with lesser $CD4^+$ cell decline in animals that were inoculated with intermediate doses of antibody. However, we noted that the lesser doses of the antibody provided complete protection against enhanced rates of the $CD4^+$ $CD95^+$ cell subpopulation in the peripheral circulation of the challenged animals, suggesting that the reshaping MAb might be able to control the activation of peripheral $CD4^+$ T cells in animals by its passive transfer. Although the number of monkeys enrolled in this study was limited, it remains noteworthy that a single inoculation with KD-247, even at a suboptimal dose for viral protection, appeared to be effective for maintaining $CD4^+$ T cells in monkeys inoculated with virus. Since it has been previously reported that the limited effect of neutralizing antibody may be related to the rapid appearance of an escape mutant in infected individuals, high titers of neutralization activity should be generated in the passively immunized animals (25, 33, 44). In our preliminary study, we isolated the escape mutant from the neutralization resistance virus $HIV\text{-}1_{JR\text{-}FL}$ in the presence of KD-247: at passage 8 of the culture in the presence of 1,000 μg/ml KD-247, one amino acid substitution, GPGR to GPER, was identified in the V3 tip (K. Yoshimura et al., unpublished results). Collectively, these results suggest that KD-247 shows clinical promise both for passive immunization and as a strategy for preventing viral spread in phenotype-matched HIV-infected individuals.

ACKNOWLEDGMENTS

We thank Richard M. Krause and Malcolm Martin, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD; Susan Zolla-Pazner, New York University School of Medicine, New York, NY; and Ruth Connor, Aaron Diamond AIDS Research Center, New York, NY, for their helpful discussions and revision of the manuscript.

This work was supported by the Panel on AIDS of the US-Japan Cooperative Medical Science Program and the Health Science Foundation, Japan.

REFERENCES

1. **Ami, Y., Y. Izumi, K. Matsuo, K. Someya, M. Kanekiyo, S. Horibata, N. Yoshino, K. Sakai, K. Shinohara, S. Matsumoto, T. Yamada, S. Yamazaki, N. Yamamoto, and M. Honda.** 2005. Priming-boosting vaccination with recombinant *Mycobacterium bovis* bacillus Calmette-Guérin and a nonreplicating vaccinia virus recombinant leads to long-lasting and effective immunity. J. Virol. **79:**12871–12879.
2. **Baba, T. W., V. Liska, R. Hofmann-Lehmann, J. Vlasak, W. Xu, S. Ayehunie, L. A. Cavacini, M. R. Posner, H. Katinger, G. Stiegler, B. J. Bernacky, T. A. Rizvi, R. Schmidt, L. R. Hill, M. E. Keeling, Y. Lu, J. E. Wright, T. C. Chou, and R. M. Ruprecht.** 2000. Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection. Nat. Med. **6:**200–206.
3. **Burton, D. R., J. Pyati, R. Koduri, S. J. Sharp, G. B. Thornton, P. W. Parren, L. S. Sawyer, R. M. Hendry, N. Dunlop, P. L. Nara, M. Lamacchia, E. Garratty, E. R. Stiehm, Y. J. Bryson, Y. Cao, J. P. Moore, D. D. Ho, and C. F. Barbas III.** 1994. Efficient neutralization of primary isolates of HIV-1 by a recombinant human monoclonal antibody. Science **266:**1024–1027.
4. **Cardoso, R. M., M. B. Zwick, R. L. Stanfield, R. Kunert, J. M. Binley, H. Katinger, D. R. Burton, and I. A. Wilson.** 2005. Broadly neutralizing anti-HIV antibody 4E10 recognizes a helical conformation of a highly conserved fusion-associated motif in gp41. Immunity **22:**163–173.
5. **Cecilia, D., V. N. Kewalramani, J. O'Leary, B. Volsky, P. Nyambi, S. Burda, S. Xu, S. R. Littman, and S. Zolla-Pazner.** 1998. Neutralization profiles of primary human immunodeficiency virus type 1 isolates in the context of coreceptor usage. J. Virol. **72:**6988–6996.
6. **Chen, Z., A. Luckay, D. L. Sodora, P. Telfer, P. Reed, A. Gettie, J. M. Kanu, and R. F. Sadek.** 1997. Human immunodeficiency virus type 2 (HIV-2) seroprevalence and characterization of a distinct HIV-2 genetic subtype from the natural range of simian immunodeficiency virus-infected sooty mangabeys. J. Virol. **71:**3953–3960.
7. **Chujoh, Y., K. Matsuo, H. Yoshizaki, T. Nakasatomi, K. Someya, Y. Okamoto, S. Naganawa, S. Haga, H. Yoshikura, A. Yamazaki, S. Yamazaki, and M. Honda.** 2002. Cross-clade neutralizing antibody production against human immunodeficiency virus type 1 clade E and B′ strains by recombinant *Mycobacterium bovis* BCG-based candidate vaccine. Vaccine **20:**797–804.
8. **Durda, P. J., L. Bacheler, P. Clapham, A. M. Jenoski, B. Leece, T. J. Matthews, A. McKnight, R. Pomerantz, M. Rayner, and K. J. Weinhold.** 1990. HIV-1 neutralizing monoclonal antibodies induced by a synthetic peptide. AIDS Res. Hum. Retrovir. **6:**1115–1123.

8a. **Eda, Y., M. Takizawa, T. Murakami, H. Maeda, K. Kimachi, H. Yonemura, S. Koyanagi, K. Shiosaki, H. Higuchi, K. Makizumi, T. Nakashima, K. Osatomi, S. Tokiyoshi, S. Matsushita, N. Yamamoto, and M. Honda.** 2006. Sequential immunization with V3 peptides from primary human immunodeficiency virus type 1 produces cross-neutralizing antibodies against primary isolates with a matching narrow-neutralization sequence motif. J. Virol. **80:**5552–5562.

9. **Emini, E. A., W. A. Schleif, J. H. Nunberg, A. J. Conley, Y. Eda, S. Tokiyoshi, S. D. Putney, S. Matsushita, K. E. Cobb, C. M. Jett, J. W. Eichberg, and K. K. Murthy.** 1992. Prevention of HIV-1 infection in chimpanzees by gp120 V3 domain-specific monoclonal antibody. Nature **355:**728–730.
10. **Gorny, M. K., J. Y. Xu, S. Karwowska, A. Buchbinder, and S. Zolla-Pazner.** 1993. Repertoire of neutralizing human monoclonal antibodies specific for the V3 domain of HIV-1 gp120. J. Immunol. **150:**635–643.
11. **Hattori, T., K. Shiozaki, Y. Eda, S. Tokiyoshi, S. Matsushita, H. Inaba, M. Fujimaki, T. Meguro, K. Yamada, M. Honda, K. Nishikawa, and K. Takatsuki.** 1991. Characteristics of the principal neutralizing determinant of HIV-1 prevalent in Japan. AIDS Res. Hum. Retrovir. **7:**825–830.
12. **Honda, M., K. Matsuo, T. Nakasone, Y. Okamoto, H. Yoshizaki, K. Watanabe, Y. Fukushima, W. Sugiura, S. Haga, Y. Katsura, K. Kitamura, H. Tasaka, K. Komuro, T. Yamada, T. Asano, A. Yamazaki, and S. Yamazaki.** 1995. Protective immune responses induced by secretion of a chimeric soluble protein from a recombinant *Mycobacterium bovis* bacillus Calmette-Guérin vector candidate vaccine for human immunodeficiency virus type 1 in small animals. Proc. Natl. Acad. Sci. USA **92:**10693–10697.

13. **Javaherian, K., A. J. Langlois, C. McDanal, K. L. Ross, L. I. Eckler, C. L. Jellis, A. T. Profy, J. R. Rusche, D. P. Bolognesi, S. D. Putney, and T. J. Matthews.** 1989. Principal neutralizing domain of the human immunodeficiency virus type 1 envelope protein. Proc. Natl. Acad. Sci. USA **86:**6768–6772.
14. **Joag, S. V., Z. Li, L. Foresman, E. B. Stephens, L. J. Zhao, I. Adany, D. M. Pinson, H. M. McClure, and O. Narayan.** 1996. Chimeric simian/human immunodeficiency virus that causes progressive loss of $CD4^+$ T cells and AIDS in pig-tailed monkeys. J. Virol. **70:**3189–3197.
15. **Karlsson, G. B., M. Halloran, J. Li, I. W. Park, R. Gomila, M. K. Reimann, S. A. Iliff, N. L. Letvin, and J. Sodroski.** 1997. Characterization of molecularly cloned simian-human immunodeficiency viruses causing rapid $CD4^+$ lymphocyte depletion in rhesus monkeys. J. Virol. **71:**4218–4225.
16. **Laman, J. D., M. M. Schellekens, Y. H. Abacioglu, G. K. Lewis, M. Tersmette, R. A. Fouchier, J. P. Langedijk, E. Claasen, and W. J. Boersma.** 1992. Variant-specific monoclonal and group-specific polyclonal human immunodeficiency virus type 1 neutralizing antibodies raised with synthetic peptides from the gp120 third variable domain. J. Virol. **66:**5175.
17. **LaRosa, G. J., J. P. Davide, K. Weinhold, J. A. Waterbury, A. T. Profy, J. A. Lewis, A. J. Langlois, G. R. Dreesman, R. N. Boswell, P. Shadduck, L. H. Holley, M. Karplus, D. P. Bolognesi, T. J. Matthews, E. A. Emini, and S. D. Putney.** 1990. Conserved sequence and structural elements in the HIV-1 principal neutralizing determinant. Science **249:**932–935.
18. **Los Alamos Database and Analysis Staff.** 2003. Part II. HIV-1/SIVepz complete genome alignments, p. 123–317. In T. Leitner, B. Foley, B. Hahn, P. A. Marx, F. McCutchan, J. W. Mellors, S. Wolinksy, and B. Korber (ed.), HIV Sequence Compendium 2003. Publication LA-UR 04–7420. Los Alamos National Laboratory, Los Alamos, N.Mex.
19. **Lu, Y., M. S. Salvato, C. D. Pauza, J. Li, J. Sodroski, K. Manson, M. Wyand, N. Letvin, S. Jenkins, N. Touzjian, C. Chutkowski, N. Kushner, M. LeFaile, L. G. Payne, and B. Roberts.** 1996. Utility of SHIV for testing HIV-1 vaccine candidates in monkeys. J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. **12:**99–106.
20. **Mascola, J. R., G. Stiegler, T. C. VanCott, H. Katinger, C. B. Carpenter, C. E. Hanson, H. Beary, D. Hayes, S. S. Frankle, D. L. Birx, and M. G. Lewis.** 2000. Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. Nat. Med. **6:**207–210.
21. **Mascola, J. R., M. G. Lewis, G. Stiegler, D. Harris, T. C. VanCott, D. Hayes, M. K. Louder, C. R. Brown, C. V. Sapan, S. S. Frankel, Y. Lu, M. L. Robb, H. Katinger, and D. L. Birx.** 1999. Protection of macaques against pathogenic simian/human immunodeficiency virus 89.6PD by passive transfer of neutralizing antibodies. J. Virol. **73:**4009–4018.
22. **Mathews, T. J.** 1994. Dilemma of neutralizing resistance of HIV-1 field isolates and vaccine development. AIDS Res. Hum. Retrovir. **10:**633–636.
23. **Matsushita, S., H. Maeda, K. Kimachi, Y. Eda, Y. Maeda, T. Murakami, S. Tokiyoshi, and K. Takatsuki.** 1992. Characterization of a mouse/human chimeric monoclonal antibody (Cβ1) to a principal neutralizing domain of the human immunodeficiency virus type 1 envelope protein. AIDS Res. Hum. Retrovir. **8:**1107–1115.
24. **Muster, T., F. Steindl, M. Purtscher, A. Trkola, G. Himmler, F. Rükler, and H. Katinger.** 1993. Conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. J. Virol. **67:**6642–6647.
25. **Narayan, S. V., S. Mukherjee, F. Jia, Z. Li, C. Wang, L. Foresman, C. McCormick-Davis, E. B. Stephens, S. V. Joag, and O. Narayan.** 1999. Characterization of a neutralization-escape variant of SHIVKU-1, a virus that causes acquired immune deficiency syndrome in pig-tailed macaques. Virology **256:**54–63.
26. **Nishimura, Y., T. Igarashi, N. Haigwood, R. Sadjadpour, R. J. Plishka, A. Buckler-White, R. Shibata, and M. A. Martin.** 2002. Determination of a statistically valid neutralization titer in plasma that confers protection against simian-human immunodeficiency virus challenge following passive transfer of high-titered neutralizing antibodies. J. Virol. **76:**2123–2130.
27. **Okamoto, Y., Y. Eda, A. Ogura, S. Shibata, T. Amagai, Y. Katsura, T. Asano, K. Kimachi, K. Makizumi, and M. Honda.** 1998. In SCID-hu mice, passive transfer of a reshaping antibody prevents infection and atrophic change of medulla in human thymic implant due to intravenous inoculation of primary HIV-1 isolate. J. Immunol. **160:**69–76.
28. **Palker, T. J., M. E. Clark, A. J. Langlois, T. J. Matthews, K. J. Weinhold, R. R. Randall, D. P. Bolognesi, and B. F. Haynes.** 1988. Type-specific neutralization of the human immunodeficiency virus with antibodies to env-encoded synthetic peptides. Proc. Natl. Acad. Sci. USA **85:**1932–1936.
29. **Parren, P. W., P. A. Marx, A. J. Hessell, A. Luckay, J. Harouse, C. Cheng-Mayer, J. P. Moore, and D. R. Burton.** 2001. Antibody protects macaques against vaginal challenge with a pathogenic R5 simian/human immunodeficiency virus at serum levels giving complete neutralization in vitro. J. Virol. **75:**8340–8347.
30. **Piatak, M., Jr., K. C. Luk, B. Williams, and J. D. Lifson.** 1993. Quantitative competitive polymerase chain reaction for accurate quantitation of HIV DNA and RNA species. BioTechniques **14:**70–81.
31. **Prince, A. M., H. Reesink, D. Pascual, B. Horowitz, I. Hewlett, K. K. Murthy, K. E. Cobb, and J. W. Eichberg.** 1991. Prevention of HIV infection by passive immunization with HIV immunoglobulin. AIDS Res. Hum. Retrovir. **7:**971–973.
32. **Reimann, K. A., J. T. Li, G. Voss, C. Lekutis, K. Tenner-Racz, P. Racz, W. Lin, D. C. Montefiori, D. E. Lee-Parritz, Y. Lu, R. G. Collman, J. Sodroski, and N. L. Letvin.** 1996. An *env* gene derived from a primary human immunodeficiency virus type 1 isolate confers high in vivo replicative capacity to a chimeric simian/human immunodeficiency virus in rhesus monkeys. J. Virol. **70:**3198–3206.
33. **Richman, D. D., T. Wrin, S. J. Little, and C. J. Petropoulos.** 2003. Rapid evolution of the neutralizing antibody response to HIV type 1 infection. Proc. Natl. Acad. Sci. USA **100:**4144–4149.
34. **Sakai, K., K. Shinohara, E. Takahashi, Y. Izumi, Y. Ami, Y. Sasaki, Y. Suzaki, S. Ando, T. Nakasone, and M. Honda.** 2001. Molecular cloning of a pathogenic simian-human immunodeficiency virus for HIV/AIDS monkey model, p. 84. Proceedings of the Sixth International Congress on AIDS in Asia and the Pacific. Melbourne, Australia.
35. **Sasaki, Y., Y. Ami, K. Shinohara, E. Takahashi, S. Ando, K. Someya, Y. Suzaki, T. Nakasone, and M. Honda.** 2000. Induction of CD95 ligand expression on $CD8^+$ T-lymphocyte correlates with HLA-DR expression and contributes to apoptosis of CD95-upregulated $CD4^+$ T-cells in monkeys by infection with a pathogenic simian/human immunodeficiency virus. Clin. Exp. Immunol. **121:**1–10.
36. **Shibata, R., T. Igarashi, N. Haigwood, A. Buckler-White, R. Ogert, W. Ross, R. Wiley, M. W. Cho, and M. Martin.** 1999. Neutralizing antibody directed against the HIV-1 envelope glycoprotein can completely block HIV-1/SIV chimeric virus infections of macaque monkeys. Nat. Med. **5:**204–210.
37. **Shinohara, K., K. Sakai, S. Ando, Y. Ami, N. Yoshino, E. Takahashi, K. Someya, Y. Suzaki, T. Nakasone, Y. Sasaki, M. Kaizu, Y. Lu, and M. Honda.** 1999. A highly pathogenic simian/human immunodeficiency virus with genetic changes in cynomolgus monkey. J. Gen. Virol. **8:**1231–1240.
38. **Someya, K., D. Cecilia, Y. Ami, T. Nakasone, K. Matsuo, S. Burda, H. Yamamoto, N. Yoshino, M. Kaizu, S. Ando, K. Okuda, S. Zolla-Pazner, S. Yamazaki, N. Yamamoto, and M. Honda.** 2005. Vaccination of rhesus monkeys with recombinant *Mycobacterium bovis* bacillus Calmette-Guérin Env V3 elicits neutralizing antibody-mediated protection against simian-human immunodeficiency virus with a homologous but not a heterologous V3 motif. J. Virol. **79:**1452–1462.
39. **Someya, K., Y. Ami, T. Nakasone, Y. Izumi, K. Matsuo, S. Horibata, K.-Q. Xin, H. Yamamoto, K. Okuda, N. Yamamoto, and M. Honda.** 2006. Induction of positive cellular and humoral immune responses by a prime-boost vaccine encoded with simian immunodeficiency virus gag/pol. J. Immunol. **176:**1784–1795.
40. **Takeuchi, H., Y. Suzuki, M. Tatsumi, H. Hoshino, E. S. Daar, and Y. Koyanagi.** 2002. Isolation and characterization of an infectious HIV type 1 molecular clone from a patient with primary infection. AIDS Res. Hum. Retrovir. **18:**1127–1133.
41. **Trkola, A., A. B. Pomales, H. Yuan, B. Korber, P. J. Maddon, G. P. Allaway, H. Katinger, C. F. Barbas III, D. R. Burton, D. D. Ho, and J. P. Moore.** 1995. Cross-clade neutralization of primary isolate of human immunodeficiency virus type 1 by human monoclonal antibodies and tetrameric CD4-IgG. J. Virol. **69:**6609–6617.
42. **Trkola, A., H. Kuster, P. Rusert, B. Joos, M. Fischer, C. Leemann, A. Manrique, M. Huber, M. Rehr, A. Oxenius, R. Weber, G. Stiegler, B. Vcelar, H. Katinger, L. Aceto, and H. F. Gunthard.** 2005. Delay of HIV-1 rebound after cessation of antiretroviral therapy through passive transfer of human neutralizing antibodies. Nat. Med. **11:**615–622.
43. **Trkola, A., M. Purtscher, T. Muster, C. Ballaun, A. Buchacher, N. Sullivan, K. Srinivasan, J. Sodroski, J. P. Moore, and H. Katinger.** 1996. Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. J. Virol. **70:**1100–1108.
44. **Wei, X., J. M. Decker, S. Wang, H. Hui, J. C. Kappes, X. Wu, J. F. Salazar-Gonzalez, M. G. Salazar, J. M. Kilby, M. S. Saag, N. L. Komarova, M. A. Nowak, B. H. Hahn, P. D. Kwong, and G. M. Shaw.** 2003. Antibody neutralization and escape by HIV-1. Nature **422:**307–312.

44a. **World Health Organization.** 2004. WHO laboratory biosafety manual, 3rd ed. World Health Organization, Geneva, Switzerland.

45. **Yamanaka, T., Y. Fujimura, S. Ishimoto, A. Yoshioka, M. Konishi, N. Narita, J. Mimaya, T. Meguro, T. Nakasone, Y. Okamoto, H. Yoshizaki, K. Yamada, and M. Honda.** 1997. Correlation of titer of antibody to principal neutralizing domain of HIV MN strain with disease progression in Japanese hemophiliacs seropositive for HIV type 1. AIDS Res. Hum. Retrovir. **13:**317–326.

JOURNAL OF VIROLOGY, Nov. 2006, p. 10683–10691 Vol. 80, No. 21
0022-538X/06/$08.00+0 doi:10.1128/JVI.01009-06

De Novo Human T-Cell Leukemia Virus Type 1 Infection of Human Lymphocytes in NOD-SCID, Common γ-Chain Knockout Mice[▽]

Paola Miyazato,[1] Jun-ichirou Yasunaga,[1] Yuko Taniguchi,[1] Yoshio Koyanagi,[2] Hiroaki Mitsuya,[3] and Masao Matsuoka[1]*

Laboratory of Virus Immunology[1] and Laboratory of Virus Pathogenesis,[2] Institute for Virus Research, Kyoto University, Kyoto 606-8507, Japan, and Department of Hematology and Department of Infectious Diseases, Graduate School of Medicine, Kumamoto University, Kumamoto 860-8556, Japan[3]

Received 17 May 2006/Accepted 21 August 2006

Human T-cell leukemia virus type 1 (HTLV-1) is the etiologic agent of adult T-cell leukemia, a disease that is triggered after a long latency period. HTLV-1 is known to spread through cell-to-cell contact. In an attempt to study the events in early stages of HTLV-1 infection, we inoculated uninfected human peripheral blood mononuclear cells and the HTLV-1-producing cell line MT-2 into NOD-SCID, common γ-chain knockout mice (human PBMC-NOG mice). HTLV-1 infection was confirmed with the detection of proviral DNA in recovered samples. Both $CD4^+$ and $CD8^+$ T cells were found to harbor the provirus, although the latter population harbored provirus to a lesser extent. Proviral loads increased with time, and inverse PCR analysis revealed the oligoclonal proliferation of infected cells. Although *tax* gene transcription was suppressed in human PBMC-NOG mice, it increased after in vitro culture. This is similar to the phenotype of HTLV-1-infected cells isolated from HTLV-1 carriers. Furthermore, the reverse transcriptase inhibitors azidothymidine and tenofovir blocked primary infection in human PBMC-NOG mice. However, when tenofovir was administered 1 week after infection, the proviral loads did not differ from those of untreated mice, indicating that after initial infection, clonal proliferation of infected cells was predominant over de novo infection of previously uninfected cells. In this study, we demonstrated that the human PBMC-NOG mouse model should be a useful tool in studying the early stages of primary HTLV-1 infection.

Human T-cell leukemia virus type 1 (HTLV-1) was the first retrovirus shown to be related to human diseases (21, 44), including adult T-cell leukemia (ATL) (50, 51, 58) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (16, 43). The infectivity of free virions is much lower than that of infected cells: transmission is cell mediated (8). Glucose transporter 1 has been identified as an HTLV-1 receptor (35). After infected cells form virological synapses with uninfected cells, the viral genome is transferred into uninfected cells (23). Hence, a salient feature of HTLV-1 infection is that this virus transmits in a cell-to-cell fashion. After infection, HTLV-1 facilitates cell-to-cell transmission by forcing the proliferation of infected cells via the actions of its accessory genes.

In the early stage of HTLV-1 infection, accessory genes including *p12*, *p30*, *p13*, and *HBZ*, have been reported to be important for in vivo proliferation of infected cells (3, 5, 22, 47). The gene product p12 plays a critical role by releasing calcium from the endoplasmic reticulum to activate nuclear factor of activated T cell-mediated transcription (2). In addition, p12 enhances lymphocyte-associated antigen-1-mediated cell adhesion, which might facilitate cell-to-cell transmission of HTLV-1 (29), and downmodulates the expression of major histocompatibility complex class I antigens (26). p30 has been reported to suppress viral gene transcription by different mechanisms (41). Other functions of p30 have been also demonstrated, such as the enhancement of the transcription of cellular genes associated with cell proliferation (38, 64). In addition, the *tax* gene is believed to play a central role in proliferation of infected cells by its pleiotropic actions (14, 17, 63). On the other hand, Tax-expressing cells are rapidly eliminated in vivo, since the Tax protein is a major target antigen of cytotoxic T lymphocytes (CTLs) (4, 27). In ATL cells, Tax expression has been shown to be suppressed by several mechanisms (52), strongly suggesting that the loss of Tax expression might be advantageous at the stage of leukemia (36). These studies reveal that the host immune system plays an important role in limiting the proliferation of infected cells. During the long latency period that spans decades, this immune pressure selects those clones with enough alterations to become malignant, eventually leading to the development of ATL.

In vivo studies of HTLV-1 infection have been carried out mainly by inoculating virus-producing or HTLV-1-immortalized cell lines into different animal species (32). Rabbits proved to be an effective model for HTLV-1 infection (1, 65). In addition, monkeys and rats have been used to analyze the in vivo proliferation of HTLV-1-infected cells (7, 55). Furthermore, immunodeficient mouse strains were also able to engraft some HTLV-1-immortalized cell lines (13, 24). These animal models are useful for studying the infection or testing therapeutic agents. However, the early steps of primary HTLV-1 infection remain uninvestigated due to the lack of in vivo experiments using human lymphocytes.

The NOD-SCID (nonobese diabetic-severe combined immunodeficiency), common γ-chain knockout (NOG) mouse was shown to be an excellent recipient for transplantation of

* Corresponding author. Mailing address: Laboratory of Virus Immunology, Institute for Virus Research, Kyoto University, Shogoin Kawahara-cho 53, Sakyo-ku, Kyoto 606-8507, Japan. Phone: 81-75-751-4048. Fax: 81-75-751-4049. E-mail: mmatsuok@virus.kyoto-u.ac.jp.

▽ Published ahead of print on 30 August 2006.

human cells due to multiple immune dysfunctions (9, 25, 60). We report here the primary infection of human lymphocytes in this newly developed mouse strain and characterize the infection by measuring proviral load as well as determining the clonality pattern. Furthermore, we tested whether the existing antiretroviral drugs azidothymidine (AZT) and tenofovir blocked primary infection in this mouse model. This small animal model allows us to better understand the mechanism of HTLV-1 infection.

MATERIALS AND METHODS

Cells. Peripheral blood mononuclear cells (PBMC) were isolated from healthy blood donors by Ficoll-Paque Plus (Pharmacia, Uppsala, Sweden) density gradient centrifugation. MT-2, an HTLV-1-producing cell line (61), was used as the source of virus in all the experiments. MT-2 cells were treated with 50 μg/ml of mitomycin C (MMC) (Kyowa, Tokyo, Japan) for 30 min at 37°C in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics and washed four times with culture medium prior to inoculation into mice. PBMC of 14 healthy donors were used in the experiments. For in vitro cytotoxicity assays, PBMC were stimulated with phytohemagglutinin (PHA) (Sigma, St. Louis, Mo.) prior to use.

Mice. The NOG strain of mice, which was generated by backcross matings of C57BL/6J-γc^{null} mice and NOD/Shi-SCID mice, is homozygous for the SCID mutation and the interleukin 2Rγ allelic mutation. It was previously reported to present multiple immunological dysfunctions that include the absence of T, B, and NK cells and also impaired activity of dendritic cells (25). Mice were purchased from the Central Institute of Experimental Animals (Kanagawa, Japan) and were maintained in microisolator cages under specific-pathogen-free conditions in the animal facility of the Institute for Virus Research, Kyoto University (Kyoto, Japan). Mice were 6 to 7 weeks old at the time of the human PBMC transfer.

Transplantation of human PBMC in NOG mice and infection with HTLV-1. A total of 10^7 human PBMC were injected intraperitoneally into each mouse, producing chimeric mice, which we will refer to as hu-PBMC-NOG mice. Three days later, the mice were inoculated intraperitoneally with MMC-treated MT-2 cells (10^3 or 10^4 cells/mouse). Spleens and cells obtained from peritoneal lavage were harvested two or four weeks after injection of MT-2 cells. Human mononuclear cells were isolated by Ficoll-Paque Plus (Pharmacia) density gradient centrifugation prior to analysis. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Institute for Virus Research, Kyoto University. In each independent experiment, PBMC from a single donor were used.

Quantification of HTLV-1 proviral load. Genomic DNA was obtained from the samples by standard proteinase K treatment. To quantify the proviral load, we performed a real-time PCR as we described previously (62). The primers for exon 3 of the HTLV-1 *tax* gene were 5′-GAAGACTGTTTGCCCACCACC-3′ and 5′-TGAGGGTTGAGTGGAACGGA-3′, and the probe was 5′-CACCCG TCACGCTAACAGCCTGGCAA-3′. Genomic DNA (500 ng) was used for real-time PCR in a 50-μl reaction solution prepared with TaqMan Universal PCR master mix (Applied Biosystems, Foster City, CA). The amplification conditions were 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 15 s at 95°C followed by 60 s at 60°C. All experiments were performed and analyzed using the ABI PRISM 7700 sequence detection system (Applied Biosystems). To measure cell equivalents in the input DNA, the recombination activating gene 1 (*RAG-1*) coding sequence in each sample was also quantified by real-time PCR. The sequences of the primers for *RAG-1* exon 2 detection were 5′-CCCACCTTGG GACTCAGTTCT-3′ and 5′-CACCCGGAACAGCTTAAATTTC-3′, and the probe was 5′-CCCCAGATGAAATTCAGCACCCACATA-3′. Amplification conditions were the same as those for *tax*. The probes were labeled with fluorescent 6-carboxyfluorescein (reporter) at the 5′ end and fluorescent 6-carboxytetramethylrhodamine (quencher) at the 3′ end. All samples were analyzed in duplicate. The DNA of freshly purified ATL cells, which harbor one copy of the HTLV-1 provirus, was used as positive control, and its proviral load was given the value of 100% when used as point of comparison.

IL-PCR. In order to study the clonality of HTLV-1 infected cells in hu-PBMC-NOG mice, we performed an inverse long PCR (IL-PCR) (10). Briefly, 1 μg of genomic DNA was first digested with EcoRI (TOYOBO, Osaka, Japan) and then self-ligated with T4 DNA ligase (TOYOBO) overnight at 4°C. Circularized DNA was then linearized with MluI (TOYOBO) to prevent amplification of the proviral sequence itself. The resulting DNA was used as template for IL-PCR, performed with LA *Taq* HS (Takara Bio Inc., Otsu, Japan). Amplification conditions were as follows: 94°C for 2 min; 40 cycles of 94°C for 30 s and 64°C for 10 min; and a final extension at 72°C for 15 min, using a Robocycler thermal cycler (Stratagene, La Jolla, CA). PCR products were electrophoresed in a 1% agarose gel and were then visualized via ethidium bromide staining.

Flow cytometric analysis. T-cell subsets of splenocytes were analyzed by flow cytometry (EPICS Coulter-Beckman, Fullerton, CA). Briefly, 10^6 cells were double stained with anti-human CD4-PC5 (Immunotech, Marseille, France) or anti-human CD8-PC5 (Immunotech) and anti-human CD45RO-fluorescein isothiocyanate (FITC) (Immunotech) or anti-human CD25-R-phycoerythrin (Caltag Laboratories, Burlingame, CA). They were also stained with anti-human CD45-FITC (Immunotech) and anti-mouse CD45-phycoerythrin (Immunotech) to assess the predominance of human cells in the recovered splenocytes. Cells were also stained with anti-human CD3-FITC (Sigma) and anti-human CD19-FITC (BD Biosciences, San Jose, CA).

Purification using magnetic beads. $CD4^+$ and $CD8^+$ T cells were isolated from 10^7 whole splenocytes by using BD IMag magnetic beads (BD Biosciences) according to the manufacturer's instructions. Positive selection of these T-cell subpopulations was performed using anti-human CD4- and anti-human CD8-conjugated magnetic particles.

Reverse transcriptase PCR (RT-PCR). RNA was extracted from splenic cells at the time of sacrifice and after 24 h of in vitro culture by using TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. One microgram of total RNA was reverse transcribed by using the RNA LA PCR kit (using avian myeloblastosis virus) version 1.1 (Takara) using random primers. One microliter of cDNA was used as the PCR template. The following primers were used: 5′-CCGGCGCTGCTCTCATCCCGG-3′ and 5′-GGCCGAACATA GTCCCCCAGAG-3′ for *tax* and 5′-GCAGGGGGGAGCCAAAAGGG-3′ and 5′-TGCCAGCCCCAGCGTCAAAG-3′ for the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene. The amplification conditions were as follows: 95°C for 2 min; 40 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 2 min (for *tax*); 95°C for 3 min; 22 cycles of 95°C for 20 s, 57°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 7 min (for the GAPDH gene) in a thermal cycler (ASTEC, Fukuoka, Japan). PCR products were electrophoresed in a 2% agarose gel and visualized via ethidium bromide staining. For real-time PCR, an ABI PRISM 7500 sequence detector (Applied Biosystems) was used. Data were analyzed by a comparative cycle threshold method. The level of *tax* mRNA in the MT-1 cell line was used as a positive control and was assigned a value of 100 arbitrary units.

Sodium bisulfite treatment of genomic DNA. Sodium bisulfite treatment was performed as previously described (54). Briefly, 1 μg of genomic DNA was denatured in 0.3 N NaOH at 37°C for 15 min, and 1 μg of salmon sperm DNA was added to each sample to act as a carrier. Sodium bisulfite (pH 5.0) and hydroquinone were added to each sample to final concentrations of 3 M and 0.05 mM, respectively, and the reaction mixture was incubated at 55°C for 16 h. Samples were then desalted using the Wizard DNA cleanup system (Promega, Madison, WI). Finally, samples were desulfonated in 0.3 N NaOH at 37°C for 15 min.

COBRA. For a combined bisulfite restriction analysis (COBRA) (59), different regions of the HTLV-1 provirus were amplified from sodium bisulfite-treated genomic DNA (54). The nested PCRs were performed using FastStart *Taq* DNA polymerase (Roche, Mannheim, Germany) under the following conditions: 95°C for 5 min; 40 cycles of 30 s at 95°C, 30 s at each annealing temperature, and 30 s at 72°C; and 2 min at 72°C for a final extension. The sequences of the primers used, and their annealing temperatures are as described previously (54). The PCR products were digested for at least 4 h with TaqI restriction enzyme, which resulted in a single recognition site within each product. The digested PCR products were separated in a 3% Nusieve 3:1 agarose (BMA, Rockland, ME) gel. The intensity of each fragment was determined by using a densitograph (version 4.0; ATTO, Tokyo, Japan).

Treatment with reverse transcriptase inhibitors in mice. hu-PBMC-NOG mice were inoculated with 10^3 MMC-treated MT-2 cells 3 days after transfer of human PBMC and were then divided into three groups for treatment, with AZT (240 mg/kg of body weight/day) (Nacalai Tesque, Kyoto, Japan), tenofovir (130 mg/kg/day) (kindly provided by Gilead Sciences Inc., CA), or phosphate-buffered saline (PBS). They were treated immediately after MT-2 inoculation for 12 days and then sacrificed to recover spleens and cells from peritoneal lavage for analysis. Tenofovir and AZT were administered intraperitoneally 2 and 3 times a day, respectively. The control group was injected twice a day with PBS. In another experiment, two groups of mice received treatment with AZT for 7 days or tenofovir for 12 days beginning one week after infection with 10^4 or 10^3 MT-2 cells/mouse, respectively. Each independent experiment was performed using the PBMC from a single donor.

TABLE 1. Proviral load of mice inoculated with different numbers of MT-2 cells[a]

Donor	No. of MT-2 cells in inoculation	Proviral load (%)	
		Lavage specimen	Spleen
A	10^2	0.0	0.0
	10^3	0.3	0.0
	10^4	4.2	1.2
B	10^2	1.1	0.2
	10^3	2.5	0.4
	10^4	0.9	2.0
C	10^6	83.2	26.5
	10^6	97.9	71.7
	10^6	90.4	53.4

[a] Proviral loads of cells recovered from the peritoneal cavity and spleens 2 (for donors A and B) or 3 (for donor C) weeks after inoculation of the specified numbers of MT-2 cells are shown for mice initially receiving PBMC of three different human donors.

MTT assay. The inhibitory effects of tenofovir and AZT on cell growth were assessed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay, which is based on the reduction of MTT by metabolically active cells to a blue formazan that can be measured spectrophotometrically. PBMC of three different donors (10^5 cells/well) were cultured in the presence or absence of the RT inhibitors (serial 10-fold dilutions from 5 mM to 0.05 μM) and 20 U/ml of interleukin 2 (kindly provided by Shionogi & Co., Ltd., Osaka, Japan) in a 96-well plate for three days. Twenty microliters of MTT solution (7.5 mg/ml) was added to each well, and the plate was incubated at 37°C for 5 h. One hundred twenty microliters of the medium was removed and 100 μl of acidified isopropanol containing 4% (vol/vol) of Triton X was added to each well to dissolve the formazan crystals. Viability relative to the untreated control was determined. Drug concentrations which inhibited cell growth by 50% (i.e., 50% cytotoxic concentrations) were also calculated from these data. All assays were performed in quadruplicate.

RESULTS

De novo HTLV-1 infection of human PBMC in NOG mice. In order to establish an in vivo model for primary HTLV-1 infection of human lymphocytes, we chose NOG mice as recipients because they were proven to engraft human cells with high efficiency (25, 60). We first determined the number of MT-2 cells necessary to achieve infection in this new mouse model. We inoculated human PBMC of two different donors intraperitoneally and, three days later, injected different numbers of MMC-treated MT-2 cells, since HTLV-1 transmits efficiently only in a cell-to-cell fashion (23, 45, 61). Two weeks later, cells were recovered from the peritoneal cavity and the spleen of each mouse and proviral load was determined by real-time PCR (Table 1). A total of 10^3 MT-2 cells was enough to produce a detectable level of proviral load in both groups of NOG mice. Taking these results into account, we decided to use 10^3 or 10^4 MT-2 cells in the following experiments. Another group of mice was inoculated with 10^6 MT-2 cells and sacrificed 3 weeks later, which led to significantly increased proviral loads (Table 1).

To check the effects of different donor sources on proviral load, we inoculated PBMC from six healthy donors into NOG mice and found that the proportions of subpopulations in T and B lymphocytes did not influence proviral loads at 2 weeks after inoculation of MT-2 cells, and the proviral loads, even in mice inoculated with cells from the same donor, were variable, especially in cells from lavages (Table 2). Regarding provirus loads in spleen cells, variations were not so remarkable. In the following experiments, we used PBMC from a single donor in each experiment.

TABLE 2. Phenotypes of donor PBMC and proviral loads of cells recovered from infected hu-PBMC-NOG mice[a]

Donor	Surface markers of donor PBMC (%)[b]				Proviral load (%)[c]	
	CD3	CD4	CD8	CD19	Lavage specimen	Spleen
D	69.7	61.8	18.7	12.2	3.7	0.6
					34.0	1.4
					2.8	0.5
E	84.7	53.4	33.9	3.0	0.6	0.1
					12.6	1.0
					11.6	0.8
F	67.0	48.0	31.9	2.8	0.2	0.0
					2.7	0.2
					0.6	0.1
G	74.9	43.9	37.7	1.3	7.4	0.2
					2.8	0.6
H	80.0	62.8	18.0	1.2	2.4	0.2
					0.4	0.0
I	ND	ND	ND	ND	20.5	2.5
					0.1	0.3

[a] PBMC from the indicated donors were transferred into NOG mice, and these were sacrificed 2 weeks after inoculation of 10^4 MMC-treated MT-2 cells.
[b] The percentage of cells positive for the specified markers before transfer into mice is shown for each donor.
[c] The proviral loads of human cells recovered from peritoneal lavage and spleens of the different mice are shown as percentages, calculated as described in Materials and Methods. ND, not determined.

In order to characterize the primary infection with HTLV-1, we inoculated a group of mice with 10^4 MT-2 cells after the transfer of PBMC and analyzed them in two groups at 2 and 4 weeks postinfection (p.i.). To assess the proportions of human cells in the studied specimens, we stained recovered cells with anti-mouse-CD45 and anti-human-CD45 antibodies and analyzed them by flow cytometry. Human cells accounted for at least 85% of the recovered splenocytes two weeks after the transfer and reached more than 94% in the group analyzed at 4 weeks p.i. (data not shown). The total number of recovered human lymphocytes was much larger than the number initially inoculated. Two weeks after the transfer of 10^7 human PBMC, we were able to recover $(7.7 \pm 3.4) \times 10^7$ human cells from the spleen of MT-2-inoculated mice and $(8.1 \pm 2.7) \times 10^7$ human cells from the spleen of the control group. These results demonstrate both migration from the peritoneal cavity to the spleen and in vivo cell expansion. There was no significant difference between the numbers of recovered splenocytes from the MT-2-inoculated and the uninoculated control groups, indicating that the cell proliferation was probably due to xenogeneic stimulation. This suggests that, in the early stages, many cells are stimulated to proliferate in the NOG mouse environment regardless of HTLV-1 infection.

In order to confirm HTLV-1 infection, we amplified a frag-

TABLE 3. Proviral load of in vivo infected cells[a]

Mouse	Proviral load (%)	
	Lavage specimen	Spleen
2-wk group		
2W-1	3.7	0.6
2W-2	34.0	1.4
2W-3	2.8	0.5
4-wk group		
4W-1	33.6	14.1
4W-2	48.1	12.9

[a] The percentages of the proviral load, calculated by comparison with a control DNA as described in Materials and Methods are shown for cells recovered from abdominal lavage and cells isolated from spleens of MT-2-inoculated hu-PBMC-NOG mice.

TABLE 4. Proviral load in $CD4^+$ and $CD8^+$ T cells[a]

Mouse	Proviral load (%)	
	$CD4^+$	$CD8^+$
2-wk group		
2W-1	0.6	0.7
2W-2	4.5	1.1
2W-3	1.2	0.4
4-wk group		
4W-1	14.9	7.8
4W-2	19.9	13.6

[a] Human $CD4^+$ and $CD8^+$ T cells were purified from 10^7 splenocytes of mice sacrificed at 2 or 4 weeks p.i. with the use of magnetic beads. Proviral load was determined by real-time PCR as described in Materials and Methods.

ment of the HTLV-1 pX region by using PCR, and proviral DNA was detected in the cells recovered from the MT-2-inoculated groups of hu-PBMC-NOG mice (data not shown). These PCR products were not derived from contamination of cellular DNA of MT-2 cells, since a PCR specific for one of HTLV-1 integration sites in MT-2 did not detect the provirus (data not shown). Splenocytes tended to have a lower proviral load than cells recovered from the peritoneal cavity. However, the proviral load in the 4-week group was generally greater than that from the 2-week group, suggesting the continuous proliferation of infected cells and propagation of the virus in this mouse model (Table 3).

Significant increase in the memory $CD4^+$ T-cell population after HTLV-1 infection. Although HTLV-1 is known to infect many types of cells in vivo (31), the majority of HTLV-1-infected cells are $CD4^+$ memory T cells (46, 62). To determine the effect of HTLV-1 infection on subpopulations of lymphocytes, we studied the expression of surface molecules by flow cytometry. Two weeks after infection, there was a significant increase in the cell population expressing CD4 and CD45RO molecules in the infected group compared to that in the control group (Fig. 1), suggesting that in the infected group of mice, memory $CD4^+$ T cells proliferated. This finding is consistent with observations with HTLV-1 carriers (62). The proviral loads in $CD4^+$ and $CD8^+$ splenic T cells were determined by real-time PCR (Table 4). As previously reported for HTLV-1 carriers, $CD8^+$ T cells were also found to contain the provirus, but to a lesser extent than $CD4^+$ T cells (39, 62). Nevertheless, proviral load tended to increase with time in both subpopulations of T cells (Table 4).

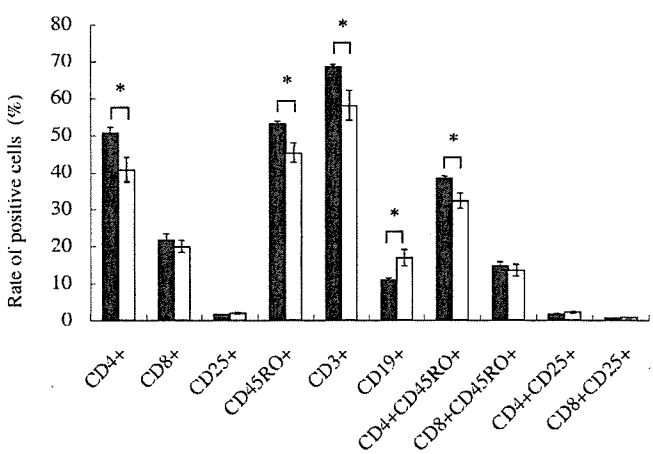

FIG. 1. Surface marker analysis of splenocytes in hu-PBMC-NOG mice. Splenocytes were isolated from hu-PBMC-NOG mice with or without HTLV-1 infection, and their surface markers were analyzed by flow cytometry. Splenocytes were recovered at 2 weeks p.i. The percentages of cells positive for various surface molecules are shown for MT-2-inoculated hu-PBMC-NOG mice (black bars) and uninfected controls (open bars). Values are means ± standard deviations from groups of three mice. *, $P < 0.05$ (Student's t test).

Polyclonal proliferation of HTLV-1-infected cells. In HTLV-1 carriers, polyclonal proliferation of HTLV-1 infected cells has been detected (10). Therefore, the clonality of HTLV-1-infected cells in hu-PBMC-NOG mice was analyzed by IL-PCR. We analyzed the same DNA samples in triplicate. When the same bands are detected in all three reactions, it means that the number of such clones is high. On the other hand, the stochastic results suggest that these clones are minor in vivo. As shown in Fig. 2, multiple bands were detected by IL-PCR at the 2-week time point, indicating an early polyclonal proliferation. At the 4-week time point, the number of bands increased, as did the intensity of bands corresponding to major clones, suggesting that both the numbers of clones and cell numbers of major clones increased (Fig. 2). We further confirmed the presence of different clones in the same mouse by determining the integration sites of the provirus in the human cells (data not shown).

Profile of proviral DNA methylation in primary HTLV-1 infection. Proviral DNA methylation appears to begin at the internal sequences, such as the *gag*, *pol*, and *env* regions (54), and accumulates in vivo. DNA methylation is thought to disturb viral gene transcription when the 5′ long terminal repeat (LTR) is methylated by inhibiting the binding of transcriptional factors (6). We analyzed the DNA methylation status of the proviral DNA in the cells recovered from the mice (Fig. 3). In the 2-week group, none of the three samples tested presented methylation in the *gag*, *pol*, or 5′ LTR regions. However, in the cells recovered from two mice after 4 weeks, the *gag* regions from both mice were partially methylated, and the *pol* region from one of the two mice was methylated. These results coincide with our previous findings that CpG motifs within the proviral sequence of HTLV-1 are methylated in a progressive manner, starting from internal regions and then spreading to the 5′ and 3′ ends of the provirus (54).

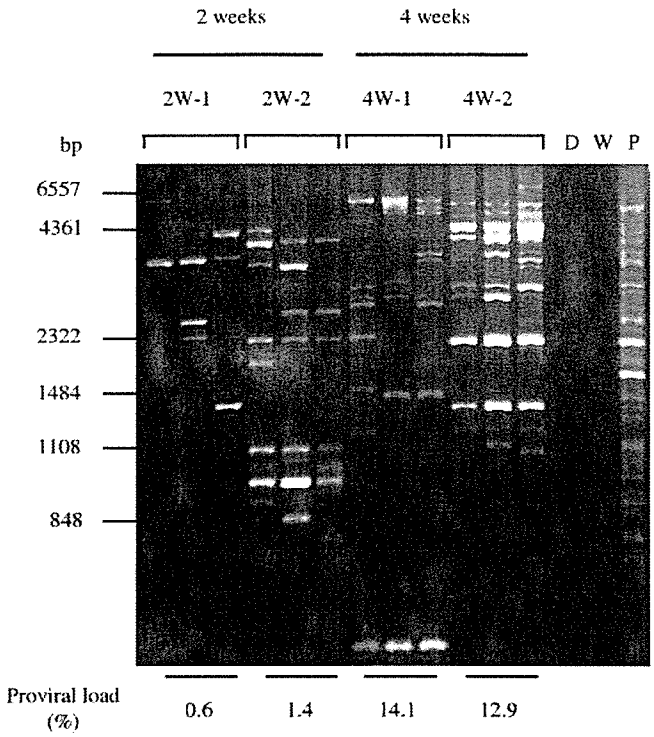

FIG. 2. Polyclonal proliferation of HTLV-1-infected cells in the spleens of hu-PBMC-NOG mice (2W-1, 2W-2, 4W-1, and 4W-2). Genomic DNA was isolated from recovered splenocytes and analyzed by IL-PCR as described in Materials and Methods. IL-PCR was performed in triplicate for each DNA sample. Genomic DNA was recovered from splenocytes at 2 or 4 weeks after injection of MT-2 cells. D, DNA of donor PBMC before inoculation; W, water; P, positive control (DNA from PBMC of an HTLV-1 carrier). In addition, proviral load was quantified by real-time PCR as described in Materials and Methods and is shown as a relative percentage.

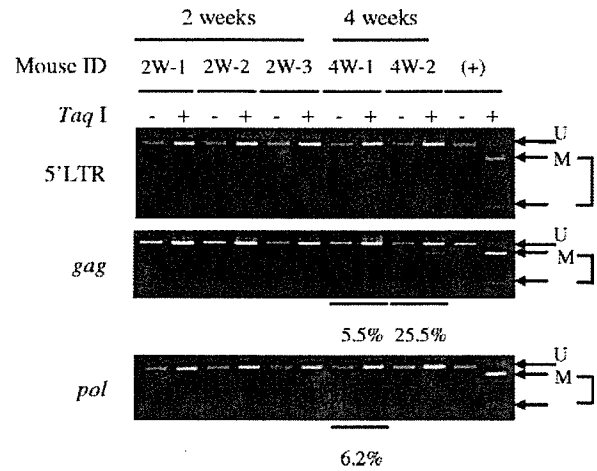

FIG. 3. DNA methylation of HTLV-1 provirus. hu-PBMC-NOG mice were sacrificed 2 or 4 weeks after inoculation of MT-2 cells, and DNA methylation in the 5′ LTR, *gag*, and *pol* regions was studied by a COBRA assay. (+), positive control; U, intact fragment (unmethylated CpG); M, digested fragments (methylated CpG). Percentages of DNA methylation were calculated by densitography according to the following formula (with the variables as described above): [M/(U + M)] × 100.

Suppression of *tax* gene transcription in the NOG mouse model. The viral protein Tax is believed to play an important role in the proliferation of infected cells due to its pleiotropic functions (63). However, its expression in vivo has not been detected in most ATL patients (52). When ATL cells are transferred to culture ex vivo, Tax expression can be recovered (21, 30, 57). Viral gene transcription is also suppressed in PBMC of HAM/TSP patients, as well as asymptomatic HTLV-1 carriers (19, 28). We performed an RT-PCR in order to detect *tax* mRNA in the spleens of infected hu-PBMC-NOG mice sacrificed 2 weeks p.i. (Fig. 4). Transcripts of the *tax* gene were undetectable in two of the three mice when cells were recovered, while the remaining one showed a low level of expression. In all three cases, there was an increase of *tax* gene transcription after 24 h of culture in vitro, even without changes in the proviral load (Fig. 4). Since this phenomenon occurs even in hu-PBMC-NOG mice, a factor(s) other than the host immune system must be involved in the suppression of *tax* gene transcription in vivo.

Effect of antiretroviral agents on HTLV-1 infection. It is well known that HTLV-1 is transmitted through sexual intercourse (49), breast feeding (48), and blood transfusions (42), and for transmission, cell-to-cell contact is thought to be essential. Due to the low capacity of cell-free virus to infect (8, 11), accidental exposures were not thought to confer a high risk of infection, and no prophylactic therapy has been considered. However, the prevalence of HTLV-1 carriers among drug abusers shows that we do need to develop strategies to prevent viral transmission. A previous in vitro study reported that AZT was able to inhibit new HTLV-1 infection of human lymphocytes (37). In addition, it has been reported that tenofovir efficiently inhibited the reverse transcriptase activity of HTLV-1 (20). In order to assess whether a preventive antiretroviral treatment would prove useful in cases of accidental HTLV-1 exposure, we treated hu-PBMC-NOG mice with two reverse transcriptase inhibitors, AZT and tenofovir. The treatment started as soon as MT-2 cells were injected and continued for 12 days. Proviral DNA was undetectable by real-time PCR in the groups of mice treated with AZT or tenofovir (Table 5). Mice seemed to tolerate the treatment without evident signs of toxicity. In the cases where weight loss was seen, it did not exceed 6% of the weight at the time treatment was started (data not shown). However, the number of human cells recovered from spleens of mice receiving AZT treatment was lower than those of the other two groups (Table 5), which indicates that this drug might be also interfering in the proliferation of transferred PBMC. In in vitro assays, we analyzed the cytotoxic effects of AZT and tenofovir on PHA-stimulated human PBMC derived from three different donors. We found that, in a range of concentrations from 5 mM to 0.05 μM, AZT was more toxic than tenofovir when used in incubations for 3 days (Fig. 5). The 50% cytotoxic concentration of AZT was 0.297 ± 0.169 mM, while that of tenofovir was higher than 5 mM. These results indicate that the cytotoxic effect of AZT contributes to suppression of the number of transferred human lymphocytes in our mouse in addition to inhibition of reverse transcriptase.

Clonal expansion of infected cells takes place even in the early stages of primary HTLV-1 infection. It remains undeter-

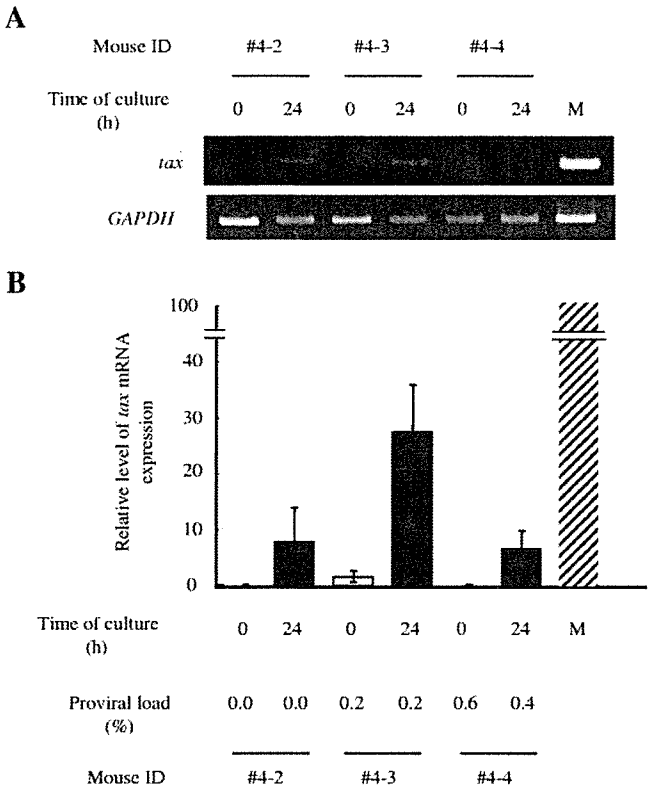

FIG. 4. Transcription of the *tax* gene increases after in vitro culture. Splenocytes of hu-PBMC-NOG mice inoculated with 10^4 MT-2 cells were recovered 2 weeks after infection. Transcription of the *tax* gene was quantified by semiquantitative PCR (A) or real-time PCR (B) at recovery and after 24 h of in vitro culture. Proviral loads for the same samples were also measured by real-time PCR. M, MT-1 cells; ID, identification number.

mined whether clonal proliferation or internal continuous contagion contributes to the increase of HTLV-1-infected cells. To answer this question, hu-PBMC-NOG mice infected with MT-2 cells were treated with tenofovir beginning 1 week after infection. Tenofovir treatment made no significant difference in HTLV-1 proviral load (Table 6), suggesting that clonal proliferation is predominant after HTLV-1 infection. The provirus loads of AZT-treated mice were lower than those of untreated mice, suggesting that the cytotoxic effect of AZT suppressed the provirus loads, as shown in Table 6.

DISCUSSION

Human immunodeficiency virus type 1 vigorously generates progeny virions through the action of its accessory genes, and the resulting free virions play an important role in its transmission, in addition to cell-to-cell transmission. In contrast, for HTLV-1, the efficiency of transmission by free virions is much lower than that via cell-to-cell contact (8), suggesting that HTLV-1 transmits primarily through the latter mechanism. To facilitate such transmission, instead of producing virions, HTLV-1 increases the number of infected cells by the actions of its accessory genes (17, 63). The finding that mother-to-infant transmission was more frequent in mothers with higher proviral loads indicates that such an increase in the number of

TABLE 5. RT inhibitors AZT and tenofovir inhibit de novo infection by HTLV-1[a]

Condition or treatment	Mouse	Proviral load (%)		Cell count (10^6)
		Lavage specimen	Spleen	
Untreated	C1	4.2	1.1	1.6
	C2	0.7	0.0	12.5
	C3	0.0	0.0	19.0
	C4	5.9	0.6	6.0
	C5	0.1	0.1	4.8
Tenofovir	T1	0.0	0.0	5.2
	T2	0.0	0.0	9.2
	T3	0.0	0.0	1.7
	T4	0.0	0.0	8.8
	T5	0.0	0.0	4.0
AZT	A1	0.0	0.0	2.6
	A2	0.0	0.0	3.6
	A3	0.0	0.0	4.5
	A4	0.0	0.0	2.2
	A5	0.0	0.0	2.3

[a] After human PBMC transfer and MT-2 inoculation (10^3 cells/mouse), mice were immediately subjected to antiretroviral therapy with AZT or tenofovir for 12 days. The control group was injected with PBS instead. Proviral loads were determined in cells recovered from the abdominal cavity and spleens. The total numbers of cells recovered from spleens are also shown.

infected cells facilitates the transmission of HTLV-1 (33). In vivo studies using animal models show that the early stage of HTLV-1 infection is controlled by accessory genes, including *p12*, *p13*, *p30*, and *HBZ* genes (3, 5, 22, 47). Thus, although in vivo studies using animal models revealed the importance of accessory genes in replication of HTLV-1 and proliferation of infected cells, the events in the early stages of in vivo transmission in human lymphocytes have not been studied yet due to the lack of an appropriate animal model. Since the metabolisms of nucleosides are quite different among animal species, it is critical to study the effect of reverse transcriptase inhibitors on HTLV-1 in human lymphocytes.

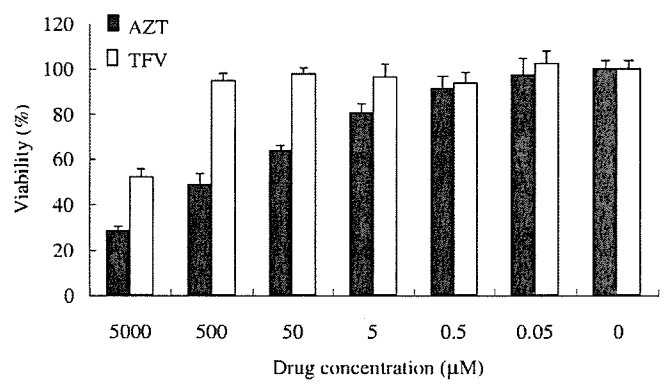

FIG. 5. Cytotoxic effects of tenofovir (TFV) and AZT in vitro. Human PBMC were stimulated with PHA for 3 days. Cells were then cultured in medium alone or medium containing the specified concentration of the indicated drug for another three days, at a density of 10^5 cells/well, in a 96-well plate. Viability was assessed by MTT assay as described in Materials and Methods. The results show the means ± standard deviations of quadruplicate measurements made in one of three representative experiments.

TABLE 6. Proviral load after treatment with tenofovir or AZT beginning one week after infection[a]

Condition or treatment	Mouse	Proviral load (%)	
		Lavage specimen	Spleen
Untreated	U11	7.4	1.7
	U12	0.1	0.0
	U13	15.4	1.9
	U14	3.8	0.7
Tenofovir	T11	0.0	0.0
	T12	0.2	0.0
	T13	15.9	1.8
	T14	14.5	2.6
Untreated	U21	0.6	0.1
	U22	ND	3.0
	U23	12.6	1.0
	U24	11.6	0.8
AZT	A21	0.0	0.0
	A22	ND	0.6
	A23	2.5	0.0
	A24	0.1	0.0

[a] After human PBMC transfer and MT-2 inoculation (10^3 cells/mouse for the tenofovir group, and 10^4 cells/mouse for the AZT group), mice were left for one week before starting treatment with tenofovir or AZT. The control groups were injected with PBS instead of the drugs. Mice were sacrificed 7 or 12 days after treatment with AZT or tenofovir, respectively. Splenocytes, as well as cells from the abdominal cavity, were recovered for analysis as described in Materials and Methods. ND, not determined.

It is widely accepted that the HTLV-1 virion per se is poorly infectious (8, 11) and that cell-to-cell transmission is more efficient both in vivo and in vitro (23, 42, 45, 61). Among drug abusers, HTLV-1 infection has been reported, indicating that HTLV-1 can be transmitted by the sharing of needles (12). Therefore, in cases of accidental exposure to HTLV-1-positive blood, preventive administration of antiretroviral drugs should be considered. In this study, we proved that the administration of a reverse transcriptase inhibitor beginning immediately after exposure can block HTLV-1 transmission. However, a delay in its administration may render it ineffective at preventing HTLV-1 transmission due to the importance of clonal expansion in the biology of this virus.

In particular, whether clonal expansion or internal continuous contagion is important in increasing the number of infected cells still remains unknown. A previous study reported that a reverse transcriptase inhibitor, lamivudine, reduced the proviral load in a patient with HAM/TSP (56), implicating internal contagion in maintaining the number of infected cells in vivo. However, another study reported that lamivudine had no definite effect on proviral load (34). In this study, administering tenofovir to block the spread of infection to new cells did not influence the proviral load in hu-PBMC-NOG mice, even though tenofovir has been reported to be more efficient in inhibiting HTLV-1 replication than lamivudine (20). Taken together, these results suggest that clonal proliferation contributes to the increase of HTLV-1-infected cells more than internal contagion even early in HTLV-1 infection. Recently, one study reported that clonality of HTLV-1-infected cells was variable after seroconversion but it became stable over time, indicating that the host immune system selected certain HTLV-1-infected clones (53). Since there is little or no host immune response to HTLV-1-infected cells in our system, it is possible that clonal proliferation of HTLV-1-infected cells is influenced by their ability to produce HTLV-1-encoded proteins, such as Tax. The factors including integration of the provirus in certain sites of the genome might also contribute to the variable proliferation of infected cells.

Viral gene transcription in HTLV-1-infected cells and ATL cells is suppressed in vivo. However, when they are cultured in vitro, transcription is rapidly recovered (54). Regarding the mechanisms of in vivo suppression, one possibility is that CTLs kill Tax-expressing cells, and the other is that nonimmune factors suppress it. The removal of $CD8^+$ T lymphocytes from PBMC derived from seropositive carriers enhanced Tax expression, suggesting that CTLs were indeed involved in inhibiting Tax expression in vivo (15, 18). On the other hand, a nonimmune factor(s) might be involved in this suppression. In this study, we showed that *tax* gene transcription was enhanced after in vitro culture. This finding is very similar to the phenomenon in carriers. It is noteworthy that in our system, there is no immune response to HTLV-1, indicating that a nonimmune factor(s) suppresses *tax* gene expression in vivo. These results suggest that both immune and nonimmune factors may be involved in the silencing of *tax* gene transcription.

Methylation of proviral DNA is regarded as a kind of host defense mechanism to suppress viral gene expression. However, HTLV-1 utilizes this epigenetic modification to escape the host immune surveillance. In cells immortalized by HTLV-1 in vitro, there was little DNA methylation in the provirus. In humans, on the other hand, DNA methylation accumulated within one year after seroconversion (54). In our system, DNA methylation was detected in the *pol* and *gag* regions 4 weeks after inoculation of MT-2 cells, indicating that HTLV-1 provirus is prone to methylation in vivo. Since *tax* gene transcription is silenced in hu-PBMC-NOG mice as shown in this study, such suppression might promote DNA methylation in vivo. On the other hand, since proliferation of HTLV-1-immortalized T lymphocytes is likely dependent on Tax expression, we speculate that cells with unmethylated provirus have growth advantages. We previously reported that histone H3 was hyperacetylated in the 5′ LTR of ATL cells without *tax* gene transcription, and such ATL cells transcribed *tax* gene within one hour after in vitro culture (54). This suggests the presence of a factor(s) inhibitory to *tax* gene transcription whose inhibition is nullified in in vitro culture. Such a mechanism, with the capacity for quickly switching on and off, would be useful for controlling *tax* gene transcription in vivo and thus enabling HTLV-1-infected cells to escape the host immune response.

In this study, we established an in vivo system for de novo infection with HTLV-1 and observed that the phenotype of HTLV-1-infected cells resembled that in the carrier state. The limitation of this in vivo system is that the long-term persistence of de novo infection in hu-PBMC-NOG mice cannot be examined, due to the graft-versus-host disease caused by implanted human lymphocytes. On the other hand, its merit is that the severe immune deficiency of this strain allows the vigorous proliferation of human lymphocytes, previously reported to be the result of a hyperactivation of the cells (40),

which enables HTLV-1 to rapidly spread by cell-to-cell contact. Therefore, this model system should be a useful tool for analyzing the events in the early stage of HTLV-1 infection in human lymphocytes.

ACKNOWLEDGMENTS

We thank Gilead Sciences Inc. for generously providing tenofovir for this study and Linda Kingsbury for excellent proofreading.

This study was supported by a grant-in-aid for scientific research from the Ministry of Education, Science, Sports, and Culture of Japan.

REFERENCES

1. **Akagi, T., I. Takeda, T. Oka, Y. Ohtsuki, S. Yano, and I. Miyoshi.** 1985. Experimental infection of rabbits with human T-cell leukemia virus type I. Jpn. J. Cancer Res. **76**:86–94.
2. **Albrecht, B., C. D. D'Souza, W. Ding, S. Tridandapani, K. M. Coggeshall, and M. D. Lairmore.** 2002. Activation of nuclear factor of activated T cells by human T-lymphotropic virus type 1 accessory protein p12^{I}. J. Virol. **76**:3493–3501.
3. **Arnold, J., B. Yamamoto, M. Li, A. J. Phipps, I. Younis, M. D. Lairmore, and P. L. Green.** 2006. Enhancement of infectivity and persistence in vivo by HBZ, a natural antisense coded protein of HTLV-1. Blood **107**:3976–3982.
4. **Bangham, C. R., and M. Osame.** 2005. Cellular immune response to HTLV-1. Oncogene **24**:6035–6046.
5. **Collins, N. D., G. C. Newbound, B. Albrecht, J. L. Beard, L. Ratner, and M. D. Lairmore.** 1998. Selective ablation of human T-cell lymphotropic virus type 1 p12I reduces viral infectivity in vivo. Blood **91**:4701–4707.
6. **Datta, S., N. H. Kothari, and H. Fan.** 2000. In vivo genomic footprinting of the human T-cell leukemia virus type 1 (HTLV-1) long terminal repeat enhancer sequences in HTLV-1-infected human T-cell lines with different levels of Tax I activity. J. Virol. **74**:8277–8285.
7. **Debacq, C., J. M. Heraud, B. Asquith, C. Bangham, F. Merien, V. Moules, F. Mortreux, E. Wattel, A. Burny, R. Kettmann, M. Kazanji, and L. Willems.** 2005. Reduced cell turnover in lymphocytic monkeys infected by human T-lymphotropic virus type 1. Oncogene **24**:7514–7523.
8. **Derse, D., S. A. Hill, P. A. Lloyd, H. Chung, and B. A. Morse.** 2001. Examining human T-lymphotropic virus type 1 infection and replication by cell-free infection with recombinant virus vectors. J. Virol. **75**:8461–8468.
9. **Dewan, M. Z., K. Terashima, M. Taruishi, H. Hasegawa, M. Ito, Y. Tanaka, N. Mori, T. Sata, Y. Koyanagi, M. Maeda, Y. Kubuki, A. Okayama, M. Fujii, and N. Yamamoto.** 2003. Rapid tumor formation of human T-cell leukemia virus type 1-infected cell lines in novel NOD-SCID/γc^{null} mice: suppression by an inhibitor against NF-κB. J. Virol. **77**:5286–5294.
10. **Etoh, K., S. Tamiya, K. Yamaguchi, A. Okayama, H. Tsubouchi, T. Ideta, N. Mueller, K. Takatsuki, and M. Matsuoka.** 1997. Persistent clonal proliferation of human T-lymphotropic virus type I-infected cells in vivo. Cancer Res. **57**:4862–4867.
11. **Fan, N., J. Gavalchin, B. Paul, K. H. Wells, M. J. Lane, and B. J. Poiesz.** 1992. Infection of peripheral blood mononuclear cells and cell lines by cell-free human T-cell lymphoma/leukemia virus type I. J. Clin. Microbiol. **30**:905–910.
12. **Feigal, E., E. Murphy, K. Vranizan, P. Bacchetti, R. Chaisson, J. E. Drummond, W. Blattner, M. McGrath, J. Greenspan, and A. Moss.** 1991. Human T cell lymphotropic virus types I and II in intravenous drug users in San Francisco: risk factors associated with seropositivity. J. Infect. Dis. **164**:36–42.
13. **Feuer, G., J. A. Zack, W. J. Harrington, Jr., R. Valderama, J. D. Rosenblatt, W. Wachsman, S. M. Baird, and I. S. Chen.** 1993. Establishment of human T-cell leukemia virus type I T-cell lymphomas in severe combined immunodeficient mice. Blood **82**:722–731.
14. **Franchini, G., R. Fukumoto, and J. R. Fullen.** 2003. T-cell control by human T-cell leukemia/lymphoma virus type 1. Int. J. Hematol. **78**:280–296.
15. **Furuta, R. A., K. Sugiura, S. Kawakita, T. Inada, S. Ikehara, T. Matsuda, and J. Fujisawa.** 2002. Mouse model for the equilibration interaction between the host immune system and human T-cell leukemia virus type 1 gene expression. J. Virol. **76**:2703–2713.
16. **Gessain, A., F. Barin, J. C. Vernant, O. Gout, L. Maurs, A. Calender, and G. de The.** 1985. Antibodies to human T-lymphotropic virus type-I in patients with tropical spastic paraparesis. Lancet **ii**:407–410.
17. **Grassmann, R., M. Aboud, and K. T. Jeang.** 2005. Molecular mechanisms of cellular transformation by HTLV-1 Tax. Oncogene **24**:5976–5985.
18. **Hanon, E., S. Hall, G. P. Taylor, M. Saito, R. Davis, Y. Tanaka, K. Usuku, M. Osame, J. N. Weber, and C. R. Bangham.** 2000. Abundant tax protein expression in CD4+ T cells infected with human T-cell lymphotropic virus type I (HTLV-I) is prevented by cytotoxic T lymphocytes. Blood **95**:1386–1392.
19. **Hanon, E., J. C. Stinchcombe, M. Saito, B. E. Asquith, G. P. Taylor, Y. Tanaka, J. N. Weber, G. M. Griffiths, and C. R. Bangham.** 2000. Fratricide among CD8(+) T lymphocytes naturally infected with human T cell lymphotropic virus type I. Immunity **13**:657–664.
20. **Hill, S. A., P. A. Lloyd, S. McDonald, J. Wykoff, and D. Derse.** 2003. Susceptibility of human T cell leukemia virus type I to nucleoside reverse transcriptase inhibitors. J. Infect. Dis. **188**:424–427.
21. **Hinuma, Y., Y. Gotoh, K. Sugamura, K. Nagata, T. Goto, M. Nakai, N. Kamada, T. Matsumoto, and K. Kinoshita.** 1982. A retrovirus associated with human adult T-cell leukemia: in vitro activation. Gann **73**:341–344.
22. **Hiraragi, H., S. J. Kim, A. J. Phipps, M. Silic-Benussi, V. Ciminale, L. Ratner, P. L. Green, and M. D. Lairmore.** 2006. Human T-lymphotropic virus type 1 mitochondrion-localizing protein p13^{II} is required for viral infectivity in vivo. J. Virol. **80**:3469–3476.
23. **Igakura, T., J. C. Stinchcombe, P. K. Goon, G. P. Taylor, J. N. Weber, G. M. Griffiths, Y. Tanaka, M. Osame, and C. R. Bangham.** 2003. Spread of HTLV-I between lymphocytes by virus-induced polarization of the cytoskeleton. Science **299**:1713–1716.
24. **Imada, K., A. Takaori-Kondo, T. Akagi, K. Shimotohno, K. Sugamura, T. Hattori, H. Yamabe, M. Okuma, and T. Uchiyama.** 1995. Tumorigenicity of human T-cell leukemia virus type I-infected cell lines in severe combined immunodeficient mice and characterization of the cells proliferating in vivo. Blood **86**:2350–2357.
25. **Ito, M., H. Hiramatsu, K. Kobayashi, K. Suzue, M. Kawahata, K. Hioki, Y. Ueyama, Y. Koyanagi, K. Sugamura, K. Tsuji, T. Heike, and T. Nakahata.** 2002. NOD/SCID/gamma(c) (null) mouse: an excellent recipient mouse model for engraftment of human cells. Blood **100**:3175–3182.
26. **Johnson, J. M., C. Nicot, J. Fullen, V. Ciminale, L. Casareto, J. C. Mulloy, S. Jacobson, and G. Franchini.** 2001. Free major histocompatibility complex class I heavy chain is preferentially targeted for degradation by human T-cell leukemia/lymphotropic virus type 1 p12^{I} protein. J. Virol. **75**:6086–6094.
27. **Kannagi, M., S. Harada, I. Maruyama, H. Inoko, H. Igarashi, G. Kuwashima, S. Sato, M. Morita, M. Kidokoro, M. Sugimoto, et al.** 1991. Predominant recognition of human T cell leukemia virus type I (HTLV-I) pX gene products by human CD8+ cytotoxic T cells directed against HTLV-I-infected cells. Int. Immunol. **3**:761–767.
28. **Kannagi, M., S. Matsushita, H. Shida, and S. Harada.** 1994. Cytotoxic T cell response and expression of the target antigen in HTLV-I infection. Leukemia **8**(Suppl. 1):S54–S59.
29. **Kim, S. J., A. M. Nair, S. Fernandez, L. Mathes, and M. D. Lairmore.** 2006. Enhancement of LFA-1-mediated T cell adhesion by human T lymphotropic virus type 1 p12I1. J. Immunol. **176**:5463–5470.
30. **Kinoshita, T., M. Shimoyama, K. Tobinai, M. Ito, S. Ito, S. Ikeda, K. Tajima, K. Shimotohno, and T. Sugimura.** 1989. Detection of mRNA for the tax1/rex1 gene of human T-cell leukemia virus type I in fresh peripheral blood mononuclear cells of adult T-cell leukemia patients and viral carriers by using the polymerase chain reaction. Proc. Natl. Acad. Sci. USA **86**:5620–5624.
31. **Koyanagi, Y., Y. Itoyama, N. Nakamura, K. Takamatsu, J. Kira, T. Iwamasa, I. Goto, and N. Yamamoto.** 1993. In vivo infection of human T-cell leukemia virus type I in non-T cells. Virology **196**:25–33.
32. **Lairmore, M. D., L. Silverman, and L. Ratner.** 2005. Animal models for human T-lymphotropic virus type 1 (HTLV-1) infection and transformation. Oncogene **24**:6005–6015.
33. **Li, H. C., R. J. Biggar, W. J. Miley, E. M. Maloney, B. Cranston, B. Hanchard, and M. Hisada.** 2004. Provirus load in breast milk and risk of mother-to-child transmission of human T lymphotropic virus type I. J. Infect. Dis. **190**:1275–1278.
34. **Machuca, A., B. Rodes, and V. Soriano.** 2001. The effect of antiretroviral therapy on HTLV infection. Virus Res. **78**:93–100.
35. **Manel, N., F. J. Kim, S. Kinet, N. Taylor, M. Sitbon, and J. L. Battini.** 2003. The ubiquitous glucose transporter GLUT-1 is a receptor for HTLV. Cell **115**:449–459.
36. **Matsuoka, M., and K. T. Jeang.** 2005. Human T-cell leukemia virus type I at age 25: a progress report. Cancer Res. **65**:4467–4470.
37. **Matsushita, S., H. Mitsuya, M. S. Reitz, and S. Broder.** 1987. Pharmacological inhibition of in vitro infectivity of human T lymphotropic virus type I. J. Clin. Investig. **80**:394–400.
38. **Michael, B., A. M. Nair, H. Hiraragi, L. Shen, G. Feuer, K. Boris-Lawrie, and M. D. Lairmore.** 2004. Human T lymphotropic virus type-1 p30II alters cellular gene expression to selectively enhance signaling pathways that activate T lymphocytes. Retrovirology **1**:39.
39. **Nagai, M., M. B. Brennan, J. A. Sakai, C. A. Mora, and S. Jacobson.** 2001. CD8(+) T cells are an in vivo reservoir for human T-cell lymphotropic virus type I. Blood **98**:1858–1861.
40. **Nakata, H., K. Maeda, T. Miyakawa, S. Shibayama, M. Matsuo, Y. Takaoka, M. Ito, Y. Koyanagi, and H. Mitsuya.** 2005. 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 gamma-chain-knocked-out AIDS mouse model. J. Virol. **79**:2087–2096.
41. **Nicot, C., M. Dundr, J. M. Johnson, J. R. Fullen, N. Alonzo, R. Fukumoto, G. L. Princler, D. Derse, T. Misteli, and G. Franchini.** 2004. HTLV-1-

encoded p30II is a post-transcriptional negative regulator of viral replication. Nat. Med. **10:**197–201.
42. **Okochi, K., and H. Sato.** 1984. Transmission of ATLV (HTLV-I) through blood transfusion. Princess Takamatsu Symp. **15:**129–135.
43. **Osame, M., K. Usuku, S. Izumo, N. Ijichi, H. Amitani, A. Igata, M. Matsumoto, and M. Tara.** 1986. HTLV-I associated myelopathy, a new clinical entity. Lancet **i:**1031–1032.
44. **Poiesz, B. J., F. W. Ruscetti, A. F. Gazdar, P. A. Bunn, J. D. Minna, and R. C. Gallo.** 1980. Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. Proc. Natl. Acad. Sci. USA **77:**7415–7419.
45. **Popovic, M., P. S. Sarin, M. Robert-Gurroff, V. S. Kalyanaraman, D. Mann, J. Minowada, and R. C. Gallo.** 1983. Isolation and transmission of human retrovirus (human T-cell leukemia virus). Science **219:**856–859.
46. **Richardson, J. H., A. J. Edwards, J. K. Cruickshank, P. Rudge, and A. G. Dalgleish.** 1990. In vivo cellular tropism of human T-cell leukemia virus type 1. J. Virol. **64:**5682–5687.
47. **Silverman, L. R., A. J. Phipps, A. Montgomery, L. Ratner, and M. D. Lairmore.** 2004. Human T-cell lymphotropic virus type 1 open reading frame II-encoded $p30^{II}$ is required for in vivo replication: evidence of in vivo reversion. J. Virol. **78:**3837–3845.
48. **Sugiyama, H., H. Doi, K. Yamaguchi, Y. Tsuji, T. Miyamoto, and S. Hino.** 1986. Significance of postnatal mother-to-child transmission of human T-lymphotropic virus type-I on the development of adult T-cell leukemia/lymphoma. J. Med. Virol. **20:**253–260.
49. **Tajima, K., S. Tominaga, and T. Suchi.** 1986. Malignant lymphomas in Japan: epidemiological analysis on adult T-cell leukemia/lymphoma. Hematol. Oncol. **4:**31–44.
50. **Takatsuki, K.** 2005. Discovery of adult T-cell leukemia. Retrovirology **2:**16.
51. **Takatsuki, K., T. Uchiyama, K. Sagawa, and J. Yodoi.** 1977. Adult T cell leukemia in Japan, p. 73–77. *In* S. Seno, F. Takaku, and S. Irino (ed.), Topic in hematology. The 16th International Congress of Hematology. Excerpta Medica, Amsterdam, The Netherlands.
52. **Takeda, S., M. Maeda, S. Morikawa, Y. Taniguchi, J. Yasunaga, K. Nosaka, Y. Tanaka, and M. Matsuoka.** 2004. Genetic and epigenetic inactivation of tax gene in adult T-cell leukemia cells. Int. J. Cancer **109:**559–567.
53. **Tanaka, G., A. Okayama, T. Watanabe, S. Aizawa, S. Stuver, N. Mueller, C. C. Hsieh, and H. Tsubouchi.** 2005. The clonal expansion of human T lymphotropic virus type 1-infected T cells: a comparison between seroconverters and long-term carriers. J. Infect. Dis. **191:**1140–1147.
54. **Taniguchi, Y., K. Nosaka, J. Yasunaga, M. Maeda, N. Mueller, A. Okayama, and M. Matsuoka.** 2005. Silencing of human T-cell leukemia virus type I gene transcription by epigenetic mechanisms. Retrovirology **2:**64.
55. **Tateno, M., N. Kondo, T. Itoh, T. Chubachi, T. Togashi, and T. Yoshiki.** 1984. Rat lymphoid cell lines with human T cell leukemia virus production. I. Biological and serological characterization. J. Exp. Med. **159:**1105–1116.
56. **Taylor, G. P., S. E. Hall, S. Navarrete, C. A. Michie, R. Davis, A. D. Witkover, M. Rossor, M. A. Nowak, P. Rudge, E. Matutes, C. R. Bangham, and J. N. Weber.** 1999. Effect of lamivudine on human T-cell leukemia virus type 1 (HTLV-1) DNA copy number, T-cell phenotype, and anti-Tax cytotoxic T-cell frequency in patients with HTLV-1-associated myelopathy. J. Virol. **73:**10289–10295.
57. **Uchiyama, T.** 1997. Human T cell leukemia virus type I (HTLV-I) and human diseases. Annu. Rev. Immunol. **15:**15–37.
58. **Uchiyama, T., J. Yodoi, K. Sagawa, K. Takatsuki, and H. Uchino.** 1977. Adult T-cell leukemia: clinical and hematologic features of 16 cases. Blood **50:**481–492.
59. **Xiong, Z., and P. W. Laird.** 1997. COBRA: a sensitive and quantitative DNA methylation assay. Nucleic Acids Res. **25:**2532–2534.
60. **Yahata, T., K. Ando, Y. Nakamura, Y. Ueyama, K. Shimamura, N. Tamaoki, S. Kato, and T. Hotta.** 2002. Functional human T lymphocyte development from cord blood CD34+ cells in nonobese diabetic/Shi-scid, IL-2 receptor gamma null mice. J. Immunol. **169:**204–209.
61. **Yamamoto, N., M. Okada, Y. Koyanagi, M. Kannagi, and Y. Hinuma.** 1982. Transformation of human leukocytes by cocultivation with an adult T cell leukemia virus producer cell line. Science **217:**737–739.
62. **Yasunaga, J., T. Sakai, K. Nosaka, K. Etoh, S. Tamiya, S. Koga, S. Mita, M. Uchino, H. Mitsuya, and M. Matsuoka.** 2001. Impaired production of naive T lymphocytes in human T-cell leukemia virus type I-infected individuals: its implications in the immunodeficient state. Blood **97:**3177–3183.
63. **Yoshida, M.** 2001. Multiple viral strategies of HTLV-1 for dysregulation of cell growth control. Annu. Rev. Immunol. **19:**475–496.
64. **Zhang, W., J. W. Nisbet, B. Albrecht, W. Ding, F. Kashanchi, J. T. Bartoe, and M. D. Lairmore.** 2001. Human T-lymphotropic virus type 1 $p30^{II}$ regulates gene transcription by binding CREB binding protein/p300. J. Virol. **75:**9885–9895.
65. **Zhao, T. M., B. Hague, D. L. Caudell, R. M. Simpson, and T. J. Kindt.** 2005. Quantification of HTLV-I proviral load in experimentally infected rabbits. Retrovirology **2:**34.

FEBS Letters 580 (2006) 2598–2602

Fumagillin suppresses HIV-1 infection of macrophages through the inhibition of Vpr activity

Nobumoto Watanabe[a,*], Yoshifumi Nishihara[a,b], Tomoyuki Yamaguchi[c], Atsushi Koito[d], Hiroyuki Miyoshi[c], Hideaki Kakeya[a], Hiroyuki Osada[a,b]

[a] *Antibiotics Laboratory, Discovery Research Institute, RIKEN, 2-1, Hirosawa, Wako, Saitama 351-0198, Japan*
[b] *Department of Applied Chemistry, Faculty of Engineering, Toyo University, 2100, Kujirai, Kawagoe, Saitama 350-8585, Japan*
[c] *Subteam for Manipulation of Cell Fate, BioResource Center, Tsukuba Institute, RIKEN, 3-1-1, Koyadai, Tsukuba, Ibaraki 305-0074, Japan*
[d] *Center for AIDS Research, Kumamoto University, 2-2-1, Honjo, Kumamoto 860-0811, Japan*

Received 17 January 2006; revised 23 March 2006; accepted 3 April 2006

Available online 19 April 2006

Edited by Hans-Dieter Klenk

Abstract HIV-1 viral protein R (Vpr) is one of the human immunodeficiency virus type 1 encoded proteins that have important roles in viral pathogenesis. However, no clinical drug for AIDS therapy that targets Vpr has been developed. Here, we have established a screening system to isolate Vpr inhibitors using budding yeast cells. We purified a Vpr inhibitory compound from fungal metabolites and identified it as fumagillin, a chemical already known to be a potent inhibitor of angiogenesis. Fumagillin not only reversed the growth inhibitory activity of Vpr in yeast and human cells, but also inhibited Vpr-dependent viral gene expression upon the infection of human macrophages.

Keywords: HIV-I; Vpr; AIDS; Small molecule; fumagillin; Cell cycle

1. Introduction

Viral protein R (Vpr) is a 96 amino acid, 14 kD nucleophilic protein that is incorporated into mature virions of human immunodeficiency virus type 1 (HIV-1). Vpr aids efficient translocation of the proviral DNA into the nucleus and is required for the HIV-1 infection of non-dividing cells such as macrophages [1–4]. Vpr is also involved in activation of viral transcription, induction of cell cycle G2 arrest and apoptosis of the host cells upon the HIV-1 infection (for recent reviews, see [5–7]). Although specific roles of these Vpr activities in viral pathogenesis and disease progression have not been elucidated, the effects of Vpr mutations found in AIDS patients suggest that Vpr is an important cytotoxic component of HIV-1 infection [8–10]. However, no Vpr targeted small molecule that can be used for AIDS therapy has yet been developed.

Fumagillin, a natural product of fungal origin, was discovered to act as a potent inhibitor of angiogenesis [11]. A semi-synthetic analog of fumagillin, TNP-470 (AGM-1470) is 50 times more active inhibitor of angiogenesis than its parent compound [11]. Since these compounds are covalent inhibitors selective for a protease, methionine aminopeptidases 2 (MetAp2), MetAp2 had been considered for the responsible molecular target for the inhibition of angiogenesis [12,13]. However, since there are two reports contradicting each other about the matter whether inhibition of angiogenesis by fumagillin is dependent on the MetAP2 activity or not, the molecular mechanism through which fumagillin inhibits angiogenesis remains to be elucidated [14,15].

In this study, we have established a screening system to isolate Vpr inhibitors. Using the system, we purified a Vpr inhibitory compound from fungal metabolites and identified it fumagillin. Fumagillin actually inhibited the HIV-1 infection of human macrophages. Our results demonstrate that fumagillin can be a lead compound for the development of the novel type of AIDS therapeutic drug that targets Vpr activity.

2. Materials and methods

2.1. Plasmids

For the expression of Vpr in yeast, *Xho*I–*Not*I fragments [16] of N-terminal FLAG-tagged HIV-1_{NL4-3}Vpr were blunted and inserted into *Bam*HI sites of copper inducible yeast expression vector, pYEX-BX (AMRAD BIOTECH, Vic., Australia). Plasmids, in which FLAG-Vpr transcription is driven in the reverse orientation under CUP1 promoter, were used as control plasmids. For the introduction of these plasmids into *URA3* cells, the *URA3* marker in pYEX-BX was changed to *HIS3*.

2.2. Yeast strains, culture and Vpr inhibitor screening

A multidrug sensitive yeast strain (MLC30) and MetAP2 deletion mutant strain (Δmap2, *map2::URA3*) were obtained from Dr. Tokichi Miyakawa (Hiroshima Univ., Higashi-Hiroshima, Japan) and Dr. Yie-Hwa Chang (St. Louis Univ. Sch. Med., MO), respectively [17,18]. For expression of Vpr, yeast cells were cultured in the SD medium (0.7% yeast nitrogen base (DIFCO), 2% glucose) containing amino acids minus selective amino acids and 0.5 mM $CuSO_4$. To silence the expression, $CuSO_4$ was removed and leucine was added to the culture. For the screening of Vpr inhibitor on agar plates, MLC30 cells with Vpr expression plasmids were cultured to log phase in the expression silencing media at 30 °C, washed, suspended in expression inducing medium at OD_{600} = 0.5 and cultured for an additional 30 min. Then, the culture was mixed with 9 volumes of expression inducing medium containing 2% agar (Phytagar, GIBCO) and 0.001% SDS, poured into plastic

*Corresponding author. Fax: +81 48 462 4669.
E-mail address: nwatanab@riken.jp (N. Watanabe).

Abbreviations: HIV-1, human immunodeficiency virus type 1; Vpr, viral protein R; MetAP2, methionine aminopeptidases 2

0014-5793/$32.00
doi:10.1016/j.febslet.2006.04.007

plates and solidified at room temperature. Paper filters (ϕ = 6 mm) containing extracts of the culture broth to be tested were put on the plates and the plates were incubated at 30 °C for several days.

2.3. Purification of fumagillin from a producing fungal strain

Fumagillin was isolated from the culture broth of a producing fungal strain using bioassay-guided purification procedures. The structure of fumagillin was determined by the physico-chemical properties, detailed ^{1}H and ^{13}C NMR analysis, and mass spectroscopy [19].

2.4. Synthesis of TNP470 and biotinylated fumagillin

TNP470 was synthesized from fumagillin as described previously [20]. Biotinylated fumagillin was synthesized by a coupling reaction via the carboxyl group of fumagillin using an activated biotin reagent (Pierce). The structures of TNP470 and biotinylated fumagillin were determined by their physico-chemical properties, detailed ^{1}H and ^{13}C NMR analysis, and mass spectroscopies. Biotinylated fumagillin was confirmed to be effective in MT-Vpr1 cells although it was weaker than the original.

2.5. Human cell culture and immunological techniques

Cell culture, synchronization and FACS analysis of MT-Vpr1 cells, a HeLa derived cell line with zinc inducible Vpr expression plasmids, and immunological detection of Vpr in the cells were as described [16]. Human embryonic kidney (HEK) 293 cells expressing SV40 large T antigen (293T) were propagated in DMEM medium supplemented with 10% FCS. Human primary monocytes and differentiated macrophages were obtained from peripheral blood mononuclear cells of healthy donors as described [21].

2.6. Preparation of viruses

To generate the single-round replication incompetent luciferase reporter virus stocks (NL-Luc-$E^{-}R^{+}$ or NL-Luc-$E^{-}R^{-}$)[2], 293T cells were co-transfected with the proviral DNAs (obtained from Dr. Nathaniel Landau through the AIDS Research and Reference Reagent Program) and plasmids encoding vesicular stomatitis virus envelope protein (pCMV-VSV-G-RSV-Rev). Culture supernatants were harvested at 60 h after the transfection and titrated.

2.7. Infectivity assays

Primary macrophages in 24 well plates were inoculated with VSV-G pseudotyped reporter viruses (NL-Luc-$E^{-}R^{+}$(VSV-G) or NL-Luc-$E^{-}R^{-}$(VSV-G); 1.5 ng of $p24^{gag}$ antigen), cultured in the absence or presence of the drug (fumagillin or TNP470) for 6 days, harvested, lysed in luciferase assay substrate (Promega) and assayed for luciferase activities using Wallac ARVO SX 1420 (Perkin–Elmer).

3. Results and discussion

3.1. Isolation of Vpr inhibitors using budding yeast cells

To isolate small molecules that inhibit the activity of Vpr, we have established a screening system using budding yeast cells expressing Vpr. As shown in Fig. 1A, yeast cells with copper-inducible Vpr expression plasmids [22] were embedded in agar plates containing the inducer ($CuSO_4$). Then, paper filters containing extracts of broth from cultured microorganisms (fungi, actinomycetes or bacteria) were put on agar plates, and the plates were incubated at 30 °C for several days. Since Vpr strongly inhibits the growth of yeast cells [22], no significant growth was usually detected even after 4–5 days of incubation. However, very occasionally, significant growth could be detected surrounding the paper filters, indicating that the culture broth extracts on the filters have an activity that antagonizes the action of Vpr (Fig. 1B). As a result of our extensive screening program, we have purified the active compound and identified it as fumagillin (Fig. 1C and D), a compound known to be a potent inhibitor of angiogenesis [11]. Commercially available fumagillin (Sigma) had specific activity similar to that of our purified compound (not shown). The activity of fumagillin could also be detected when a galactose inducible system was used for Vpr expression (not shown), suggesting that this compound reverses the action of Vpr itself rather than the expression of Vpr by copper inducible system (see below).

3.2. Effect of fumagillin and TNP470 on Vpr induced cell cycle arrest in HeLa cells

Next, we examined the ability of fumagillin to antagonize Vpr function in human cells. As described above, one of the characteristic functions of Vpr in human cells is induction of the cell cycle arrest at G2 phase [23,24]. We previously established a HeLa derived cell line (MT-Vpr1) stably transfected with a zinc-inducible Vpr expression vector [16]. In this cell line, induction of Vpr expression arrests cell cycle at G2 phase in more than half of the total cells a day after addition of the inducer (Fig. 2A, Zn). When fumagillin (10 ng/ml) was added before the addition of zinc, the G2 arrested population was

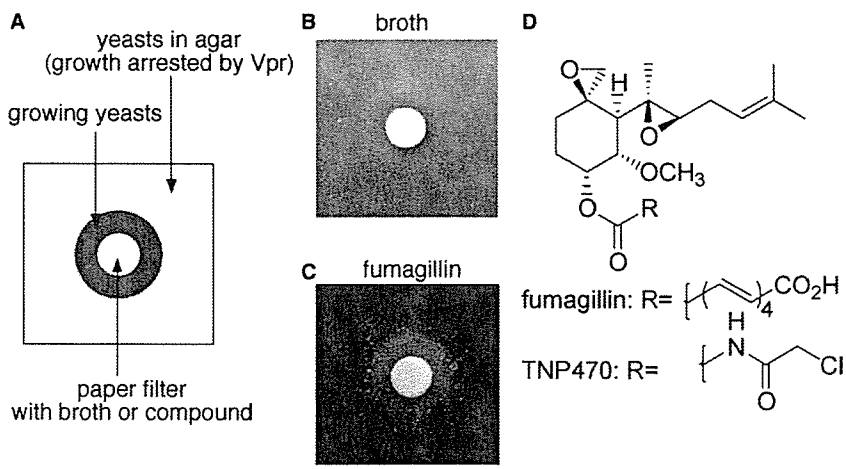

Fig. 1. Screening system to isolate Vpr inhibitors. (A) Schematic presentation of Vpr screening system. Budding yeast cells expressing Vpr were embedded in agar plates containing inducer (copper). Paper filters with broths or compounds to be tested were put on the plates. Only the yeasts surrounding filters that contain Vpr inhibitors were able to grow. (B,C) Growing yeasts surrounding filters containing 10 μl of extract from the culture broth of a fungus with Vpr inhibitory activity (B) or purified fumagillin (C; 2 mg/ml, 10 μl). Plates were incubated for 4 days at 30 °C. (D) Chemical structures of fumagillin and TNP470.

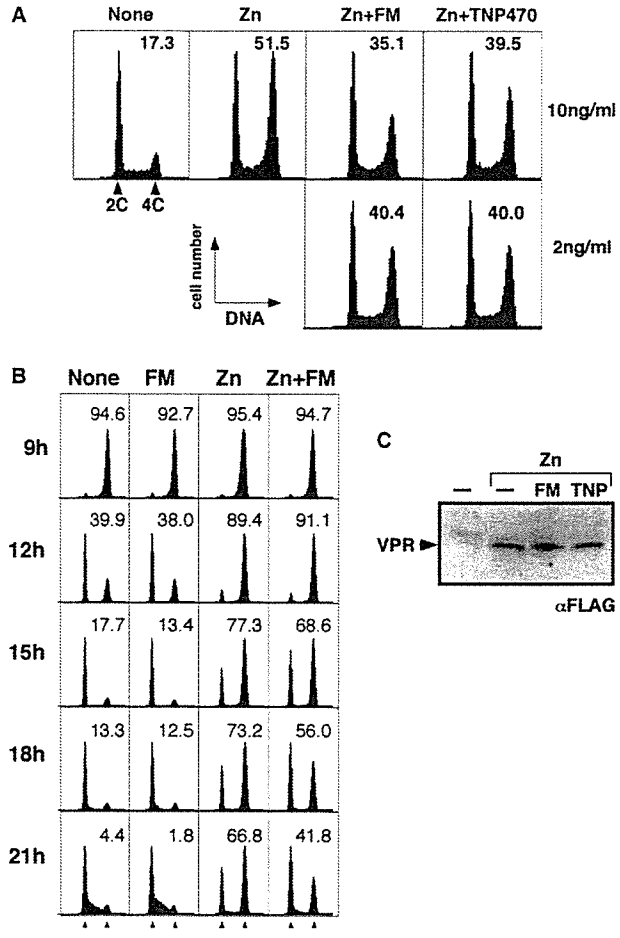

Fig. 2. Fumagillin and TNP470 inhibit Vpr activity in HeLa cells. (A) Thirty minutes before the addition of zinc, fumagillin (FM) or TNP470 was added to MT-Vpr1 cells at the concentrations described. Cells were cultured for a day in the presence or absence of zinc (Zn; 150 μM) and harvested for FACS analysis. Numbers in the figures represent the percentage of cells with 4C DNA contents. (B) Mt-Vpr1 cells were synchronized at G1/S border [16], then released in the presence or absence of zinc (Zn; 150 μM) and/or fumagillin (FM; 10 ng/ml). Zinc and fumagillin were added at 2 and 1 h before the release, respectively. Numbers in the figures represent the percentage of cells with 4C DNA contents. (C) Cells as in (A) were cultured for 6 h and harvested for western analysis to detect FLAG-Vpr expression [16]. Drugs were added at 10 ng/ml.

significantly reduced (Fig. 2A, Zn + FM). Thus, fumagillin can partially inhibit the action of Vpr in mammalian cells as well. The effect of higher doses (100 ng/ml and 1 μg/ml) of fumagillin on the action of Vpr was almost similar to that at 10 ng/ml in this system (not shown).

A synthetic analog of fumagillin, TNP470 (AGM-1470; Fig. 1D) is a more potent angiogenesis inhibitor [11]. However, the ability of TNP470 to antagonize Vpr function was similar to or less than that of fumagillin (Fig. 2A, Zn + TNP470). Thus, fumagillin seems to override Vpr-dependent cell cycle arrest in a manner different from that through which it blocks angiogenesis.

In this system, however, if fumagillin has an activity to arrest cell cycle at a phase other than G2 phase, an apparent reduction of G2 arrested cells would be observed. To examine this possibility, the effect of fumagillin on cell cycle progression was examined. After MT-Vpr1 cells were synchronized at G1/S border and released in the presence or absence of fumagillin, progression through S, G2, M and G1 was monitored by FACS analysis (Fig. 2B). In the absence of zinc, cell cycle progression was not affected by fumagillin (None and FM). When Vpr expression was induced by zinc addition, a similar fraction of cells was arrested at G2 at 12 h after the release regardless of the presence of fumagillin. But, in the presence of fumagillin, the fraction of cells arrested at G2 phase was significantly reduced at later time points (Zn and Zn + FM). These results indicate that fumagillin does not affect normal cell cycle progression but reduces the activity of Vpr to arrest the cell cycle. We have also confirmed that neither fumagillin nor TNP470 has any effect on the zinc induced Vpr expression level in MT-Vpr1 cells (Fig. 2C).

3.3. *Vpr inhibits growth of yeast cells independently from MetAP2 pathway*

Fumagillin is known to covalently bind and inhibit a protease, MetAP2 both in human and budding yeast cells [12,13]. However, since there are two reports contradicting each other about the matter whether inhibition of angiogenesis by fumagillin is dependent on the MetAP2 activity or not, the molecular mechanism through which fumagillin inhibits angiogenesis remains to be elucidated [14,15].

We examined whether MetAP2 is on the pathway for Vpr-dependent growth arrest and whether fumagillin blocks the activity of Vpr through the inhibition of MetAP2 or not. In budding yeast, the gene (*MAP2*) that encodes MetAP2 is not essential, because there is a second aminopeptidase, MetAP1, which is insensitive to fumagillin [12,13,18]. Vpr arrested the growth of Δ*map2* strain cells almost as completely as wild type, indicating that MetAP2 is not on the pathway of the Vpr dependent growth arrest (Fig. 3A). The ability of fumagillin to reverse the Vpr dependent arrest in Δ*map2* strain cells was confirmed on paper disk assay as well (data not shown). These results indicate that fumagillin abrogates Vpr function by targeting (a) molecule(s) other than MetAP2. Since the sensitivity to fumagillin and TNP470 is different for Vpr-dependent arrest and inhibition of angiogenesis, the target molecule(s) for these drugs may be different in these two systems.

3.4. *Mechanism of fumagillin to inhibit Vpr function*

Using biotinylated fumagillin, we attempted to detect any covalent or strong binding between Vpr and fumagillin. Biotinylated fumagillin was added to the lysates of MT-Vpr1 cells or yeast cells expressing FLAG-tagged Vpr. After the lysates were separated on SDS–PAGE and transferred on membrane, proteins covalently bound to fumagillin such as MetAP2 were probed with horse radish peroxidase (HRP) labelled streptavidin. Alternatively, proteins associated with biotinylated fumagillin were isolated using streptavidin conjugated agarose beads, and probed with αFLAG antibody to detect FLAG tagged Vpr. In spite of all of these attempts, we were unable to obtain any evidence for the interaction between Vpr and fumagillin (not shown). However, when point-mutations (Q3R, E25K, A30L, W54A, L64A, H71R, R73A, I74R, G75A, C76A, R80A, and R90K) were introduced into Vpr and their sensitivity to fumagillin was examined on paper disk assay, we found that the E25K mutation (the 25th glutamate of Vpr was changed to lysine) makes Vpr significantly resistant to fumagillin (Fig. 3B). Since the E25K Vpr still inhibits growth of yeast cells [25], the mechanism of fumagillin may

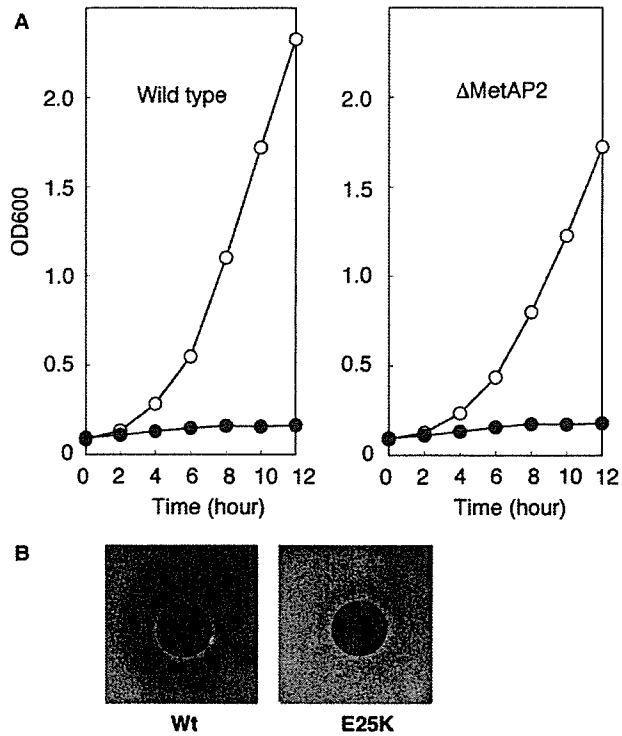

Fig. 3. Mechanism of fumagillin to abrogate the Vpr activity. (A) Vpr inhibits growth of yeast cells independently from MetAP2 activity. Δ*map2* cells (right) or its isogenic control cells (left) were cultured in the presence (closed circle) or absence (open circle) of the Vpr expression. The growth of yeast cells was monitored with the absorbance at 600 nm. (B) E25K mutation makes Vpr resistant to fumagillin. Yeast cells with wild type Vpr (left) or E25K mutated Vpr were embedded in agar plates as in Fig. 1. Paper filters with 20 μg of fumagillin were put on the plates and incubated for 3 days at 30 °C. Photographs were taken with translucent light to increase sensitivity.

be directly on Vpr rather than on a downstream pathway. The precise mechanism through which the E25K mutation renders Vpr resistant to fumagillin is not clear, but it is possible that fumagillin interacts directly (albeit too weakly to detect) with Vpr at residues surrounding E25.

3.5. *Inhibition of Vpr-dependent viral gene expression by fumagillin or TNP470*

Vpr is required for efficient replication of HIV-1 in non-dividing cells such as macrophages [2–4]. During the HIV-1 life cycle, Vpr functions after entry and reverse transcription, yet prior to, or at the time of, proviral transcription [2]. Thus we examined the effect of fumagillin on the proviral transcription upon the infection using an *env*-deficient HIV-1 vector that allows only a single round of infection. Wild type or frame-shifted Vpr-containing, *env*-deficient HIV-1 reporter vector in which Nef has been replaced by the luciferase gene (NL-Luc-R^+ or NL-Luc-R^-, respectively) [2] was used to infect primary human macrophages (Fig. 4A and B). Luciferase activity, determined 6 days after infection, was about 4 times higher from the Vpr^+ virus than that from the Vpr^- virus, indicating that Vpr is required for efficient expression of virally encoded genes in macrophages [2]. When fumagillin or TNP470 was added at the time of infection, luciferase expression from the Vpr^+ virus but not from the Vpr^- virus was inhibited in a dose-dependent manner. Under these experimental condition,

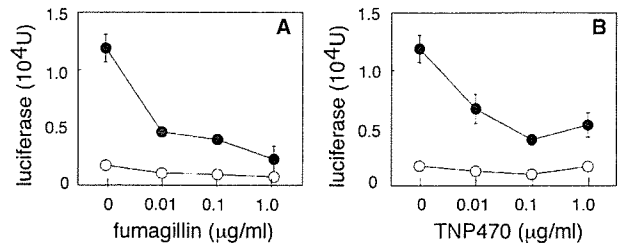

Fig. 4. Fumagillin and TNP470 inhibit Vpr dependent proviral gene expression. (A,B) Macrophages were infected with HIV-1 reporter vector with wild type (closed circle) or truncated (open circle) Vpr and cultured in the presence of fumagillin (A) or TNP470 (B). The proviral gene expression was monitored by the luciferase activity 6 days after the infection using luciferase assay substrate (Promega).

we could not see any sign of toxicity of 1 μg/ml of fumagillin for the macrophages under microscope indicating that the inhibition of viral gene expression in Vpr^+ infected cells is due to the inhibition of Vpr by these drugs rather than to some non-specific toxicity of them. Taken together, our results show that fumagillin or TNP470 suppresses the HIV-1 replication in macrophages through inhibition of Vpr-dependent viral gene expression.

3.6. *Concluding remarks*

Because it is now evident that Vpr's contribution to the pathogenesis of HIV-1 infection in vivo is crucial, Vpr has been proposed to be an attractive target for developing novel therapeutic strategies for AIDS therapy. Our results show that fumagillin and its derivatives can be used as a new type of AIDS therapeutic drug, which targets Vpr. In this context, it should be noted that fumagillin and TNP470 are already used clinically to treat Kaposi's sarcoma or microsporidiosis in AIDS patients with successful results [26,27], although the effects of these drugs on the viral replication have not been reported. Thus, the day when the fumagillin-derived compounds can be used clinically to prevent HIV-1 replication may come sooner than expected.

Acknowledgments: We thank T. Miyakawa, Y.-H. Chang and N.R. Landau for reagents; C. Tsutsui and A. Masumoto for assistance; members of RIKEN Antibiotics Laboratory for discussions; T. Hunter for critical reading of the manuscript; M. Watanabe and Y. Ikawa for encouragement. This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT) and by the Chemical Biology Research Project (RIKEN).

References

[1] Heinzinger, N.K., Bukinsky, M.I., Haggerty, S.A., Ragland, A.M., Kewalramani, V., Lee, M.A., Gendelman, H.E., Ratner, L., Stevenson, M. and Emerman, M. (1994) The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. Proc. Natl. Acad. Sci. USA 91, 7311–7315.
[2] Connor, R.I., Chen, B.K., Choe, S. and Landau, N.R. (1995) Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes. Virology 206, 935–944.
[3] Vodicka, M.A., Koepp, D.M., Silver, P.A. and Emerman, M. (1998) HIV-1 Vpr interacts with the nuclear transport pathway to promote macrophage infection. Genes Dev. 12, 175–185.
[4] Subbramanian, R.A., Kessous-Elbaz, A., Lodge, R., Forget, J., Yao, X.J., Bergeron, D. and Cohen, E.A. (1998) Human

immunodeficiency virus type 1 Vpr is a positive regulator of viral transcription and infectivity in primary human macrophages. J. Exp. Med. 187, 1103–1111.
[5] Andersen, J.L. and Planelles, V. (2005) The role of Vpr in HIV-1 pathogenesis. Curr. HIV Res. 3, 43–51.
[6] Le Rouzic, E. and Benichou, S. (2005) The Vpr protein from HIV-1: distinct roles along the viral life cycle. Retrovirology 2, 11.
[7] Muthumani, K., Choo, A.Y., Premkumar, A., Hwang, D.S., Thieu, K.P., Desai, B.M. and Weiner, D.B. (2005) Human immunodeficiency virus type 1 (HIV-1) Vpr-regulated cell death: insights into mechanism. Cell Death Differ. 12, 962–970.
[8] Goh, W.C., Rogel, M.E., Kinsey, C.M., Michael, S.F., Fultz, P.N., Nowak, M.A., Hahn, B.H. and Emerman, M. (1998) HIV-1 Vpr increases viral expression by manipulation of the cell cycle: a mechanism for selection of Vpr in vivo. Nat. Med. 4, 65–71.
[9] Somasundaran, M., Sharkey, M., Brichacek, B., Luzuriaga, K., Emerman, M., Sullivan, J.L. and Stevenson, M. (2002) Evidence for a cytopathogenicity determinant in HIV-1 Vpr. Proc. Natl. Acad. Sci. USA 99, 9503–9508.
[10] Lum, J.J., Cohen, O.J., Nie, Z., Weaver, J.G., Gomez, T.S., Yao, X.J., Lynch, D., Pilon, A.A., Hawley, N., Kim, J.E., Chen, Z., Montpetit, M., Sanchez-Dardon, J., Cohen, E.A. and Badley, A.D. (2003) Vpr R77Q is associated with long-term nonprogressive HIV infection and impaired induction of apoptosis. J. Clin. Invest. 111, 1547–1554.
[11] Ingber, D., Fujita, T., Kishimoto, S., Sudo, K., Kanamaru, T., Brem, H. and Folkman, J. (1990) Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumour growth. Nature 348, 555–557.
[12] Sin, N., Meng, L., Wang, M.Q., Wen, J.J., Bornmann, W.G. and Crews, C.M. (1997) The anti-angiogenic agent fumagillin covalently binds and inhibits the methionine aminopeptidase, MetAP-2. Proc. Natl. Acad. Sci. USA 94, 6099–6103.
[13] Griffith, E.C., Su, Z., Turk, B.E., Chen, S., Chang, Y.H., Wu, Z., Biemann, K. and Liu, J.O. (1997) Methionine aminopeptidase (type 2) is the common target for angiogenesis inhibitors AGM-1470 and ovalicin. Chem. Biol. 4, 461–471.
[14] Kim, S., LaMontagne, K., Sabio, M., Sharma, S., Versace, R.W., Yusuff, N. and Phillips, P.E. (2004) Depletion of methionine aminopeptidase 2 does not alter cell response to fumagillin or bengamides. Cancer Res. 64, 2984–2987.
[15] Bernier, S.G., Taghizadeh, N., Thompson, C.D., Westlin, W.F. and Hannig, G. (2005) Methionine aminopeptidases type I and type II are essential to control cell proliferation. J. Cell. Biochem. 95, 1191–1203.
[16] Watanabe, N., Yamaguchi, T., Akimoto, Y., Rattner, J.B., Hirano, H. and Nakauchi, H. (2000) Induction of M-phase arrest and apoptosis after HIV-1 Vpr expression through uncoupling of nuclear and centrosomal cycle in HeLa cells. Exp. Cell Res. 258, 261–269.
[17] Miyamoto, Y., Machida, K., Mizunuma, M., Emoto, Y., Sato, N., Miyahara, K., Hirata, D., Usui, T., Takahashi, H., Osada, H. and Miyakawa, T. (2002) Identification of *Saccharomyces cerevisiae* isoleucyl-tRNA synthetase as a target of the G1-specific inhibitor Reveromycin A. J. Biol. Chem. 277, 28810–28814.
[18] Li, X. and Chang, Y.H. (1995) Amino-terminal protein processing in *Saccharomyces cerevisiae* is an essential function that requires two distinct methionine aminopeptidases. Proc. Natl. Acad. Sci. USA 92, 12357–12361.
[19] Asami, Y., Kakeya, H., Onose, R., Chang, Y.-H., Toi, M. and Osada, H. (2004) RK-805, an endothelial-cell-growth inhibitor produced by *Neosartorya* sp. and a docking model with methionine aminopeptidase-2. Tetrahedron 60, 7085–7091.
[20] Marui, S., Itoh, F., Kozai, Y., Sudo, K. and Kishimoto, S. (1992) Chemical modification of fumagillin. I. 6-*O*-acyl, 6-*O*-sulfonyl, 6-*O*-alkyl, and 6-*O*-(*N*-substituted-carbamoyl)fumagillols. Chem. Pharm. Bull. (Tokyo) 40, 96–101.
[21] Cheng-Mayer, C., Quiroga, M., Tung, J.W., Dina, D. and Levy, J.A. (1990) Viral determinants of human immunodeficiency virus type 1 T-cell or macrophage tropism, cytopathogenicity, and CD4 antigen modulation. J. Virol. 64, 4390–4398.
[22] Macreadie, I.G., Castelli, L.A., Hewish, D.R., Kirkpatrick, A., Ward, A.C. and Azad, A.A. (1995) A domain of human immunodeficiency virus type 1 Vpr containing repeated H(S/F)RIG amino acid motifs causes cell growth arrest and structural defects. Proc. Natl. Acad. Sci. USA 92, 2770–2774.
[23] Jowett, J.B., Planelles, V., Poon, B., Shah, N.P., Chen, M.L. and Chen, I.S. (1995) The human immunodeficiency virus type 1 vpr gene arrests infected T cells in the G2 + M phase of the cell cycle. J. Virol. 69, 6304–6313.
[24] He, J., Choe, S., Walker, R., Di Marzio, P., Morgan, D.O. and Landau, N.R. (1995) Human immunodeficiency virus type 1 viral protein R (Vpr) arrests cells in the G2 phase of the cell cycle by inhibiting p34cdc2 activity. J. Virol. 69, 6705–6711.
[25] Yao, X.J., Rougeau, N., Duisit, G., Lemay, J. and Cohen, E.A. (2004) Analysis of HIV-1 Vpr determinants responsible for cell growth arrest in *Saccharomyces cerevisiae*. Retrovirology 1, 21.
[26] Dezube, B.J., Von Roenn, J.H., Holden-Wiltse, J., Cheung, T.W., Remick, S.C., Cooley, T.P., Moore, J., Sommadossi, J.P., Shriver, S.L., Suckow, C.W. and Gill, P.S. (1998) Fumagillin analog in the treatment of Kaposi's sarcoma: a phase I AIDS Clinical Trial Group study. AIDS Clinical Trial Group No. 215 Team. J. Clin. Oncol. 16, 1444–1449.
[27] Didier, E.S. (2005) Microsporidiosis: an emerging and opportunistic infection in humans and animals. Acta Trop. 94, 61–76.

Resistance profile of a neutralizing anti-HIV monoclonal antibody, KD-247, that shows favourable synergism with anti-CCR5 inhibitors

Kazuhisa Yoshimura[a], Junji Shibata[a], Tetsuya Kimura[a], Akiko Honda[a], Yosuke Maeda[b], Atsushi Koito[a], Toshio Murakami[c], Hiroaki Mitsuya[d] and Shuzo Matsushita[a]

Background: The high-affinity humanized monoclonal antibody (MAb) KD-247 reacts with a tip region in gp120-V3 and cross-neutralizes primary isolates with a matching neutralization sequence motif.

Methods: We induced an HIV-1 variant that was resistant to KD-247 by exposing the JR-FL virus to increasing concentrations of KD-247 in PM1/CCR5 cells, which expressed high levels of CCR5 *in vitro*. We determined the amino acid sequence of the gp120-encoding region of the JR-FL escape mutant from KD-247. To confirm that this substitution was responsible for the KD-247-resistance, a single-round replication assay was performed. We further evaluated the anti-HIV-1 interactions between KD-247 and various CCR5 inhibitors *in vitro*.

Results: At passage 8 of the culture in the presence of 1000 μg/ml KD-247, one amino acid substitution, Gly to Glu at position 314 (G314E), was identified in the V3-tip of gp120. A pseudotyped virus with the G314E mutation was highly resistant to KD-247. Unexpectedly, this mutant virus was sensitive to CCR5 inhibitors, RANTES, recombinant human soluble CD4 (rsCD4) and an anti-CCR5 MAb, but resistant to an anti-CD4 MAb, compared with the wild-type virus. We also found that combinations of KD-247 and CCR5 inhibitors were highly synergistic.

Conclusions: The present data suggest that KD-247 has certain advantages for possible passive immunotherapy. They are: high concentrations of KD-247 are needed for viral acquisition of KD-247 resistance; the escape variants are more sensitive to CCR5 inhibitors and rsCD4; and there are high levels of synergism between KD-247 and CCR5 inhibitors at all concentrations tested.

AIDS 2006, **20**:2065–2073

Keywords: HIV-1, KD-247, anti-V3 monoclonal antibody, broadly neutralizing, CCR5 inhibitor, synergism

From the [a]Division of Clinical Retrovirology and Infectious Diseases, Center for AIDS Research, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan, the [b]Department of Medical Virology, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan, the [c]The Chemo-Sero-Therapeutic Research Institute, Kyokushi, Kikuchi, Kumamoto, Japan, and the [d]Department of Internal Medicine II, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan.

Correspondence to S. Matsushita, Division of Clinical Retrovirology and Infectious Diseases, Center for AIDS Research, Kumamoto University, Kumamoto 860-0811, Japan.

Tel: +81 96 373 6536; fax: +81 96 373 6537; e-mail: shuzo@kaiju.medic.kumamoto-u.ac.jp

These data were presented at the *Thirteenth Conference on Retroviruses and Opportunistic Infections*. Denver, CO, February 2006 [abstract 506].

Note: K. Yoshimura and J. Shibata contributed equally to this work.

Received: 21 February 2006; accepted: 27 July 2006.

ISSN 0269-9370