IRF3 activation and 'deactivation' must be strictly controlled. Pin1-mediated post-translational regulation of IRF3, therefore, is likely to be important in determining the exact nature of the final immune responses against viruses or microbes.

TBK1-NAK and IKK-*i*–IKKε have been identified as IRF3 kinases responsible for the phosphorylation of the two C-terminal phospho-accepter clusters (Ser385-Ser386 and Ser396-Ser398-Ser402-Thr404-Ser405) and are indispensable for IRF3 activation[14–17]. Our results here have demonstrated that Ser339 is another important phosphorylation target for IRF3 regulation that governs the termination of IRF3-dependent transcriptional activation. Of note, the Pin1 target motif of human IRF3 is conserved in human, monkey, mouse and rat IRF7, another member of the IRF family. As IRF7 is a 'master regulator' for type I interferon expression induced by TLR7 and TLR9 stimulation[38], Pin1 might act as a critical regulator of type I interferon production by modulating both IRF3 and IRF7 signaling. Further studies are needed to fully elucidate the functions of Pin1 in the regulation of type I interferon production.

Here we have demonstrated activation-induced destabilization of IRF3 through direct engagement with Pin1. In general, degradation of transcription factors is one of the principal mechanisms that reduce or terminate transcriptional activation. This type of mechanism occurs not only during immune responses but also in many other biological phenomena. An example of this appeared in a study demonstrating that the turnover of c-Myc is under strict control by phosphorylation-dependent post-translational modification and that c-Myc deregulation results in the induction of oncogenic transformation of fibroblasts[28,39]. Phosphorylation at Ser62 of c-Myc is required for its stabilization, a phosphorylation event that is also a prerequisite for subsequent phosphorylation at Thr58 by glycogen synthase kinase-3β, which in turn leads to ubiquitination and proteasome-dependent degradation of the protein. Thus, sequential phosphorylation events are critical for regulation of the c-Myc-induced proliferation signals[28,39]. Consistent with that, substitution of Thr58 with alanine renders c-Myc more stable and oncogenic[28]. Notably, Pin1 is recruited to the phosphorylated Thr58-Pro59 motif of c-Myc and is critical in the phosphorylation-induced destabilization of c-Myc[28]. Because Pin1 is recruited to phosphorylated IRF3 after stimulation, it would be useful to determine if phosphorylation of the two clusters essential for activation of IRF3, but which do not mediate Pin1 binding, alters IRF3 stability and the efficiency of IRF3 interaction via the Pin1-binding motif on IRF3. Phosphorylation of the C-terminal phospho-accepter cluster of IRF3 is a prerequisite for MG132-sensitive reduction of IRF3 in a human cell line after infection with Sendai virus[10], although the underlying molecular mechanism was not clarified in that study. Although Pin1 regulates the protein stability of many transcription factors, it does not directly catalyze the ubiquitination or subsequent proteasome-dependent degradation of its substrates. Therefore, the identification of an IRF3-specific ubiquitin ligase or conjugating enzyme would facilitate fuller understanding of the regulation of IRF3 signaling.

METHODS

Reagents. Monoclonal anti-α-tubulin and anti-hemagglutinin (HA-7) were purchased from Sigma. Polyclonal anti-Pin1 and monoclonal anti-Myc were purchased from Cell Signaling Technology. Monoclonal anti-Pin1 was purchased from R&D Systems. Polyclonal anti-IRF3 (FL-425) was purchased from Santa Cruz Biotechnology. Polyclonal antibody to human IRF3 phosphorylated at Ser339 was elicited by immunization of a rabbit with the phosphorylated peptide N-CEGSGR(pS)PRY-C. Poly(I)•poly(C) was purchased from Amersham. Calf intestinal alkaline phosphatase was purchased from Takara Shuzo. MG132 was purchased from the Peptide Institute. Human IFN-β and the human IFN-β enzyme-linked immunosorbent assay (ELISA) kit were purchased from FujiRebio. The mouse IFN-β ELISA kit was purchased from PBL Biomedical Laboratories. All other reagents were purchased from Sigma unless indicated otherwise. Polyclonal anti-IRF3 has been described[8]. NDV, EMCV and VSV were prepared as described[5].

Plasmids. The expression constructs pFLAG-CMV1-hTLR3, pMX-puro, pEF1-lacZ and p125-Luc (IFN-β promoter with a luciferase (Luc) reporter) were donated by K. Fitzgerald (University of Massachusetts, Worcester, Massachusetts), T. Kitamura (University of Tokyo, Tokyo, Japan), S. Memet (Institut Pasteur, Paris, France) and T. Taniguchi (University of Tokyo, Tokyo, Japan), respectively. The pcDNA3-Pin1, pcDNA3-Pin1 W34A, pcDNA3-Pin1 K63A, pGEX-Pin1, pCMV-Myc-Ub, pEF-BOS hemagglutinin-tagged IRF3 (HA-IRF3), pEF-BOS dominant negative HA-IRF3 (58-427), pGal4-DBD, pGal4-IRF3, pUAS-Luc, pHCMV-VSV-G, pMRX-IRES-puro and pMRX-IRES-bsr constructs have been described[5,8,20,26,40,41]. The pEF-myc-cyto and pcDNA3 plasmids were purchased from Invitrogen. Construction details for the other plasmids are in the **Supplementary Methods** online.

Cells and mice. Neomycin-resistant 293-TLR3neo^r, Plat-E and U373-CD14 cells have been described[9,29,42]. The 293-TLR3 cultures were established after infection with retrovirus produced from pMX-Flag-hTLR3-puro. *Pin1*$^{-/-}$ mice have been described[20,25,26,43], as have MEFs isolated from *Pin1*$^{+/+}$ and *Pin1*$^{-/-}$ mice[26].

Reporter assays and retrovirus preparations. Cells were transfected using the FuGene6 transfection reagent (Roche) according to the manufacturer's instructions. The culture supernatant of the packaging cell line Plat-E, cotransfected with a retroviral vector and pHCMV-VSV-G, was filtered and used for infection[40]. Reporter assays were done as described[30]. Firefly luciferase activity was normalized to β-galactosidase activity.

Preparation of bone marrow–derived macrophages. Bone marrow–derived macrophages isolated from 12-week-old *Pin1*$^{+/+}$ or *Pin1*$^{-/-}$ mice were differentiated *in vitro* for 6 d. Macrophages were then left unstimulated or stimulated with poly(I)•poly(C). Secreted IFN-β was measured with the mouse IFN-β ELISA kit according to the manufacturer's guidelines.

Injection of poly(I)•poly(C) into mice. Poly(I)•poly(C) (5 μg per gram of body weight) was injected into the peritonea of 12-week-old *Pin1*$^{+/+}$ or *Pin1*$^{-/-}$ mice. Blood was drawn from the tail vein before and 10 h after treatment. Collected sera were immediately frozen. IFN-β in sera was measured with the mouse IFN-β ELISA kit according to the manufacturer's guidelines.

Immunocytochemistry and nuclear staining. Immunocytochemistry and nuclear staining with fluorescent dyes were done as described[8,26].

Immunoblot, immunoprecipitation and GST affinity assays. Immunoprecipitation and GST affinity assays were done as described[26,30]. For the ubiquitination assay, cells either treated or left untreated were suspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% (volume/volume) glycerol and 1% (volume/volume) Nonidet-P40) supplemented with 0.5 mM phenyl methyl sulfonylfluoride, 3 μg/ml of leupeptin, 10 μM MG132, 10 μM MG115, 5 mM NaF and 1 mM Na_3VO_4. Cell lysates were incubated for 2 h with anti-Myc and then were incubated for 1 h with protein G–Sepharose. Beads were washed four times with lysis buffer. SDS-PAGE and native PAGE coupled with immunoblot analysis were done as described[26,30].

Protein stability assay. Protein stability was analyzed as described[26]. After 293-TLR3 cells were transfected with the HA-IRF3 expression plasmid together with pcDNA3-HA-lacZ, they were subjected to various treatments. After the addition of cycloheximide, cells were collected at various time points. Hemagglutinin-tagged proteins were detected by immunoblot and were semiquantified with NIH image software.

Virus yield titration. MEFs or Vero cells were infected with VSV or EMCV. Virus yield in culture supernatants was determined by plaque assay as described[5].

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Note: Supplementary information is available on the Nature Immunology website.

ACKNOWLEDGMENTS

We thank K. Swanson for technical advice. Supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan (13226027 and 14406009 to N.Y.; 16659125 and 17013029 to S.Y.), the National Institutes of Health (GM58556 to K.P.L.), the Japan Society for the Promotion of Science (T.S.), the Swiss Foundation for Grants in Biology and Medicine (A.T.-K.) and the Leukemia and Lymphoma Society (A.R.).

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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JOURNAL OF VIROLOGY, June 2006, p. 5563–5570 Vol. 80, No. 11
0022-538X/06/$08.00+0 doi:10.1128/JVI.02095-05
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Anti-V3 Humanized Antibody KD-247 Effectively Suppresses Ex Vivo Generation of Human Immunodeficiency Virus Type 1 and Affords Sterile Protection of Monkeys against a Heterologous Simian/Human Immunodeficiency Virus Infection

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Received 5 October 2005/Accepted 9 March 2006

In an accompanying report (Y. Eda, 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, J. Virol. 80:5552-5562, 2006), we discuss our production of a high-affinity humanized monoclonal antibody, KD-247, by sequential immunization with V3 peptides derived from human immunodeficiency virus type 1 (HIV-1) clade B primary isolates. Epitope mapping revealed that KD-247 recognized the Pro-Gly-Arg V3 tip sequence conserved in HIV-1 clade B isolates. In this study, we further demonstrate that in vitro, KD-247 efficiently neutralizes CXCR4- and CCR5-tropic primary HIV-1 clade B and clade B′ with matching neutralization sequence motifs but does not neutralize sequence-mismatched clade B and clade E isolates. Monkeys were provided sterile protection against heterologous simian/human immunodeficiency virus challenge by the passive transfer of a single high dose (45 mg per kg of body weight) of KD-247 and afforded partial protection by lower antibody doses (30 and 15 mg per kg). Protective neutralization endpoint titers in plasma at the time of virus challenge were 1:160 in animals passively transferred with a high dose of the antibody. The antiviral efficacy of the antibody was further confirmed by its suppression of the ex vivo generation of primary HIV-1 quasispecies in peripheral blood mononuclear cell cultures from HIV-infected individuals. Therefore, KD-247 promises to be a valuable tool not only as a passive immunization antibody for the prevention of HIV infection but also as an immunotherapy for the suppression of HIV in phenotype-matched HIV-infected individuals.

Because most primary strains of human immunodeficiency virus type 1 (HIV-1) are relatively resistant to neutralization, the specificities of antibodies that confer protective immunity against it are still not understood (22). Previously, we and others (9, 31) have reported that chimpanzees can be protected against infection with the T-cell-line-adapted strain HIV-1_{IIIB} by passive transfer of either HIV immunoglobulin (Ig) (HIVIG) or anti-HIV-1_{IIIB} V3 monoclonal antibodies (MAbs). Passive administration of the anti-HIV-1 gp41 human MAb 2F5 (24) to two chimpanzees prior to challenge with primary HIV-1_{5016} resulted in a delay in plasma viremia and reduced viral load. Since the chimpanzee model is limited by the failure of HIV-1 to induce disease in these animals, a pathogenic model was developed in monkeys using a simian/human immunodeficiency virus (SHIV) strain that is capable of inducing high plasma viremia, $CD4^+$-T-cell loss, and simian AIDS (11, 14, 15, 37). Following pathogenic SHIV 89.6P challenge, Mascola and colleagues (20) previously noted a synergistic effect with the passively transferred antibody HIVIG, a MAb against membrane-proximal external region 2F5 (27), and 2G12, a glycan-dependent MAb (41). Monkeys were afforded protective immunity against pathogenic SHIV DH12 by chimpanzee HIVIG and provided sterile protection against the challenge virus when given high-dose inoculations (27, 36). However, sterile protection was strain specific, and the antiserum did not bind a V3 loop peptide or block the interaction of gp120 with CD4. In several passive immunization studies using MAbs, the antibodies 2G12 and 2F5 as well as 4410, a MAb against membrane-proximal external region 4E10 (4), have been shown to inhibit SHIV in monkeys (2, 20, 21). Furthermore, human MAb b12, targeting the CD4-binding domain of gp120, has been reported to elicit complete protection against viral challenge (29) and partial protection against MAb 2G12 (22) in monkeys. Recently, passively transferred antibodies with 2G12, 2F5, and 4E10 were shown to delay the rebound of HIV-1 after the cessation of antiretroviral therapy, with that delay especially pronounced in acutely infected individuals.

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The in vivo effect of the neutralizing antibody cocktail was found to depend on 2G12 activity by escape mutant analysis (42).

It has been established that anti-V3 antibodies, induced by brief immunization protocols in animals, are capable of neutralizing HIV-1 in cell cultures and in animal challenge studies (13, 16, 27, 28). However, that capability has not been fully exploited because the V3 sequence is extremely diverse, and so the anti-V3 antibodies are extremely type specific and displayed little cross-reactivity. In the accompanying paper (8a), we describe how we sequentially immunized mice with V3 peptides derived from several different HIV-1 clade B field isolates. The antibody response could be traced to a tip sequence of the HIV-1 gp120 V3 domain, a relatively conserved motif (11, 18, 45). We reshaped anti-V3 MAb C25 into KD-247, a humanized MAb directed against the V3 tip motif Pro-Gly-Arg of the V3 domain. KD-247 cross-neutralized primary isolates with a matching neutralization sequence motif, suggesting that it could be used to overcome the previous limitations surrounding anti-V3 neutralizing antibody production by active immunization strategies.

In this study, we show that the humanized MAb KD-247 is suitable not only for use as a passive immunization antibody for the prevention of immunodeficiency virus infection but also to passively transfer antibodies for immunotherapy. Using 18 primary HIV-1 isolates, we evaluate the neutralizing capacity of KD-247. We also assess its efficacy against ex vivo generation of HIV from the peripheral blood mononuclear cells (PBMCs) of four HIV-infected individuals. Finally, we examine whether KD-247 can suppress HIV-1 replication in monkeys.

MATERIALS AND METHODS

Passive transfer of KD-247 to monkeys followed by pathogenic virus challenge. All animals used in this study were mature, cycling, male cynomolgus monkeys (*Macaca fascicularis*) from the Tsukuba Primate Center, National Institute of Infectious Diseases (NIID), Japan. They were free of known simian retroviruses, herpesviruses, bacteria, and parasites. They were housed in accordance with the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science under the Japanese Law Concerning the Protection and Management of Animals (1, 38) and were maintained in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee of NIID, Japan. Once approved by an institutional committee for biosafety level 3 experiments, these studies were conducted at the Tsukuba Primate Center, NIID, Japan, in accordance with the requirements specifically stated in the laboratory biosafety manual of the World Health Organization (44a).

The pathogenic SHIV strain C2/1 is an SHIV strain 89.6 variant isolated by in vivo passage in cynomolgus monkeys (37). The original SHIV 89.6 strain was kindly provided by Y. Lu at the Harvard AIDS Institute (Boston, MA) (19, 32). Virus stocks of SHIV C2/1 were stored at −125°C and thawed just prior to use. The challenge stock was provided by K. Shinohara of the National Institute of Infectious Diseases, Tokyo, Japan. Cynomolgus monkeys injected intravenously with SHIV C2/1 showed high levels of viremia and marked $CD4^+$-T-cell depletion within 2 weeks after inoculation (1, 34, 35, 37). Naïve monkeys were intravenously administered 0, 15, 30, or 45 mg/kg of KD-247 along with either 45 mg/kg of purified normal human immunoglobulin (Nihon Pharmaceutical Co., Tokyo, Japan) or saline. Twenty-four hours after antibody transfer, the animals were intravenously challenged with 20 50% tissue culture infective doses ($TCID_{50}$s) of SHIV C2/1.

In vitro virus neutralization assays. The primary clinical isolate $HIV\text{-}1_{MNp}$ was kindly provided by J. Sullivan of the University of Massachusetts Medical School, Worcester, MA. The virus was confirmed to be neutralization resistant (5). Laboratory-adapted $HIV\text{-}1_{89.6}$ and $HIV\text{-}1_{MN}$ were obtained from the AIDS Research and Reference Reagent Program, National Institutes of Health, Rockville, MD. GHOST cell neutralization assays were performed as described previously (5, 38). Briefly, GHOST cells expressing either CXCR4 or CCR5 coreceptors were used as targets of HIV-1 infection. The cells were then analyzed by FACSCalibur flow cytometry (Becton Dickinson, San Jose, CA). The same concentration of either purified normal human immunoglobulin consisting primarily of the IgG1 subclass (Nihon Pharmaceutical Co.) or saline was used as control.

Neutralization activities in monkey plasma were assayed by detecting the neutralizing titers in the assay measuring 100% neutralization against the challenge virus as described previously by Nishimura et al. (26). In brief, plasma samples were serially diluted and incubated with 100 $TCID_{50}$s of challenge virus, and M8166 cells were then incubated as previously described (26). The neutralization was expressed as the percent inhibition of simian immunodeficiency virus p27 antigen production in the culture supernatants (38, 39). Normal monkey plasma was used as a control.

PBMC-based virus neutralization assay. $HIV\text{-}1_{MN}$ (H9/HTLV-III MN) was kindly provided by the AIDS Research and Reference Reagent Program, National Institutes of Health, Rockville, MD (45). The WHO primary isolates 92TH002, 92TH022, 92TH023 (all clade E), and 92TH014 (clade B′) were used as virus stocks (12). The primary isolates $HIV\text{-}1_{JR\text{-}CSF}$ and the CS and JCI series of HIV-1 isolates were provided by Y. Koyanagi (40) and Y. Okamoto (27). In vitro virus neutralization assays were performed as previously described (7, 12). Neutralization titers are expressed as either the concentration of serum IgG antibody or the reciprocal of the serum dilution that yielded a 50% (50% inhibitory concentration [IC_{50}]) or 90% (IC_{90}) reduction in HIV-1 p24 production over that seen in controls using purified serum IgG from healthy individuals or preimmune mouse sera.

Ex vivo virus neutralization assays. The PBMCs of patients infected with HIV-1 were depleted of $CD8^+$ cells by magnetic separation using polystyrene beads coated with anti-CD8 MAb (Dynabeads M-450 CD8; Dynal, Oslo, Norway). The negatively selected cells were stimulated with OKT3 antibody (1 μg/ml; Jannsen-Kyowa, Tokyo, Japan) and subsequently cultured in the presence of interleukin-2 (20 U/ml; Boehringer, Mannheim, Germany) together with KD-247 (60 and 240 μg/ml). The amount of HIV-1 p24 antigen in the supernatant was determined by enzyme-linked immunosorbent assay (ELISA) (Dainabot, Tokyo, Japan). Approval by the ethical committee and written informed consent from all the human subjects were obtained according to the guidelines of the Ministry of Health, Labor, and Welfare, Japan, and to those of the Kumamoto University Medical School, Kumamoto, Japan.

Competitive PCR quantitation of SHIV RNA in plasma. Quantitative competitive reverse transcription-PCR was performed as described previously by Piatak et al. (30), with both the substitution of a different competitor RNA and a different DNA template (35). The detection limit of this assay was 500 RNA copies/ml in monkey plasma.

Flow cytometric evaluation of cell surface antigen expression and absolute cell count. Mouse MAbs conjugated with either fluorescein isothiocyanate, phycoerythrin (PE), PE-Cy5, or peridinin chlorophyll protein were used in flow cytometric analyses to detect cellular expression of monkey CD3 (NF-18; BioSource International Inc., Camarillo, CA), human CD4 (Nu-TH/I; Nichirei Co., Tokyo, Japan), CD8 (SK-1; Becton Dickinson & Co., San Jose, CA), and CD95 (CH11 and 7C11; Becton Dickinson) (30). To determine absolute cell counts, samples of whole blood were analyzed following the addition of fluorescein isothiocyanate-conjugated anti-CD3 (BioSource), PE-conjugated anti-CD4 (Becton Dickinson), and peridinin chlorophyll protein-conjugated anti-CD8 (Becton Dickinson) MAbs as previously described (35).

Plasma concentration of KD-247. HIV-1 V3 peptide-based ELISA was used for quantification of KD-247 antibody. In brief, 96-well ELISA plates (Maxisorp; Nunc A/S, Roskilde, Denmark) were coated with 100 μl of a KD-247 antigen peptide (SP1 [YNKRKRIHIGPGRAFYTTKNC]) per well in 50 mM carbonate buffer (pH 9.3) at 1 μg/ml overnight at 4°C. KD-247 was diluted to concentrations ranging from 2.5 to 40 ng/ml as a reference. Bound KD-247 was detected with a peroxidase-conjugated anti-human IgG MAb (in-house preparation; The Chemo-Sero-Therapeutic Research Institute). The concentrations of KD-247 in the plasma of monkeys were determined using a calibration curve (SOFTmax; Molecular Devices Co., Menlo Park, CA).

Statistical analysis. The plasma concentrations at various data points postdose were applied to a two-compartment model using an automatic pharmacokinetic analysis program (nonlinear least-squares method), and pharmacokinetic parameters were calculated.

RESULTS

Neutralization ability of the humanized antibody KD-247 against a panel of primary isolates as determined by a PBMC-based study. In the initial series of the study, we showed that

TABLE 1. PBMC-based neutralization of primary and laboratory isolates by KD-247[a]

Isolate	Env V3 sequence[b]		GHOST cell	KD-247		447-52D IC_{50}[c]
				IC_{90}	IC_{50}	
Laboratory isolates, clade B						
HIV-1_{MN}	CTRPNYNKRKRIHI	GPGRAFYTTKNIIGTIRQAHC	X4	1	0.1	0.1
HIV-1_{SF2}	-----N-T--G---	-------A-EK-V-D------	X4	5	1.0	1.0
HIV-$1_{89.6}$	-----N-T-R-LS-	-------ARR----D------	R5/X4	2.5	0.2	>10
Primary isolates, clade B						
HIV-1_{JR-CSF}	----SN-K--S---	---------GE---D------	R5	5	0.4	>10
HIV-1_{CS2-2}	-----N-T--S--M	---K-----GD---N----Y-	R5	>50	>50	ND
HIV-1_{CS3-5}	---I-N-T--S---	-------A-GE---N-K----	R5	10	1.4	ND
HIV-1_{CS4-4}	-I---N-T--G---	-L-- WK--A-G--N------	R5/X4	>50	>50	ND
HIV-1_{CS6-6}	--G--N-T--S-R-QR------V-IGK --NM-----		R5	>50	>50	ND
HIV-1_{CS6-8}	-I---N-T--G---	-------A-D----N------	R5	8	1.2	ND
HIV-1_{JCI-1}	----HKTI------	----------Q-E-N-----	X4	5	0.4	ND
HIV-1_{JCI-2}	----SN-T-R----	---------RQ-R-D-----	X4	4	0.2	ND
HIV-1_{JCI-3}	-----N-I--H---	---------RG -RD--K---	R5	10	0.6	ND
HIV-1_{JCI-5}	-------T--G---	------V---G -RD--K---	X4	4	0.2	ND
HIV-1_{JCI-6}	----SN-T-R----	-----S--A-Q-RGD------	X4	6	0.7	ND
HIV-1_{JCI-9}	-------T--G---	------V---G -RD--K---	R5	21	1.6	ND
HIV-1_{JCI-11}	-------TS-G-R-	-------ASER -RD--K---	R5	34	3.2	ND
HIV-1_{JCI-22}	-----N-I--H---	---------RG -RD--K---	R5	12	1.2	ND
Primary isolates, clade B′						
HIV-$1_{92TH014}$	-----N-T--S-PL	-----W---GQ---D------	R5	8	0.9	>1.5
Primary isolates, clade E						
HIV-$1_{92TH002}$	----SN-T-TS-T-	---QV--R-GD---D--K-Y-	R5	>50	>50	ND
HIV-$1_{92TH022}$	----SN-T-TS-T-	---QV--R-GD---D--K-Y-	R5	>50	>50	>10
HIV-$1_{92TH023}$	----SN-T-TS-N-	---QV--R-GD---D--K-Y-	R5	>50	>50	ND
SHIV-B						
SHIV 89.6PD	-----N-T-R-LS-	-------ARR----D------	R5/X4	5	0.5	ND
SHIV C2/1	-----N-T-E-LS-	-------ARR----D------	R5/X4	5	0.5	ND

[a] The HIV-1 sequences were confirmed by proviral DNA sequencing of virus-infected cells.
[b] Dashes indicate sequence homology to HIV-1_{MN}, and spaces represent the presence of a deletion.
[c] ND, not done.

sequential immunization with synthetic V3 peptides from representatives of primary HIV-1 clade B isolates generated cross-reactive antisera and produced a high-affinity humanized MAb, KD-247, directed against the tip of the HIV-1 V3 domain, PGR. Furthermore, the humanized antibody more effectively neutralized several primary isolates of HIV-1 clade B than did previously reported neutralization antibodies (8a, 10, 23, 27). To further analyze the divergence of the cross-neutralization ability of the antibody by a PBMC-based HIV-1 neutralization assay, we used a panel of a total of 23 immunodeficiency viruses: 18 primary isolates of HIV-1 clade B, clade B′, and clade E viruses; 3 laboratory HIV-1 clade B viruses; and 2 highly pathogenic SHIVs (Table 1). The KD-247 antibody effectively neutralized HIV-1_{MN}, HIV-1_{SF2}, and HIV-$1_{89.6}$, containing the consensus V3 sequence of HIV-1 clade B, IGPGR AFY, with an IC_{90} and IC_{50} from 1 to 5 and from 0.1 to 1.0 μg/ml, respectively (Table 1, laboratory isolates, clade B). We next sought to assess whether the neutralization of primary isolates by KD-247 required a matching neutralization sequence motif. As expected, KD-247 effectively neutralized primary CCR5-tropic clade B and B′ isolates (IC_{90} and IC_{50} from 5 to 34 and from 0.4 to 3.2 μg/ml, respectively) and all four of the CXCR4-tropic clade B isolates (IC_{90} and IC_{50} from 4 to 6 and from 0.2 to 0.7 μg/ml, respectively) with matching IGPGR or V3 tip sequences. Thus, CCR5-tropic isolates with an IC_{90} of a mean concentration of neutralization antibody of 13.5 μg/ml were more than 2.8 times less sensitive to the neutralization by KD-247 than primary CXCR4-tropic isolates with a mean IC_{90} of 4.8 μg/ml. In contrast, the neutralization-resistant virus CS2-2 did not match the neutralization sequence motif, and the CS6-6 virus showed a QR insertion in the V3 tip sequence. The HIV-1 isolates containing a glutamine (Q) residue at position 20 in the V3 region, such as those of subtype E, were also resistant to neutralization by KD-247. Therefore, KD-247 effectively neutralizes both the CCR5- and CXCR4-tropic primary isolates with matching neutralization motifs.

Ex vivo suppressive effects of KD-247 on the generation of HIV-1 quasispecies from PBMCs of HIV-infected individuals. To fully assess the antiviral efficacy of KD-247, we next sought to determine whether it would suppress the generation of HIV-1 from PBMCs of HIV-infected individuals and whether it would do so as efficiently as an established anti-V3 humanized antibody, Cβ1 (23). As shown in Table 2, we investigated the effect of KD-247 at concentrations of 60 and 240 μg/ml on the ex vivo generation of HIV-1 using $CD8^+$-T-cell-depleted PBMC cultures from four Japanese individuals infected with HIV-1 clade B (Env V3 sequence in Table 2). In the presence of KD-247 at concentrations of 60 and 240 μg/ml, the gener-

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

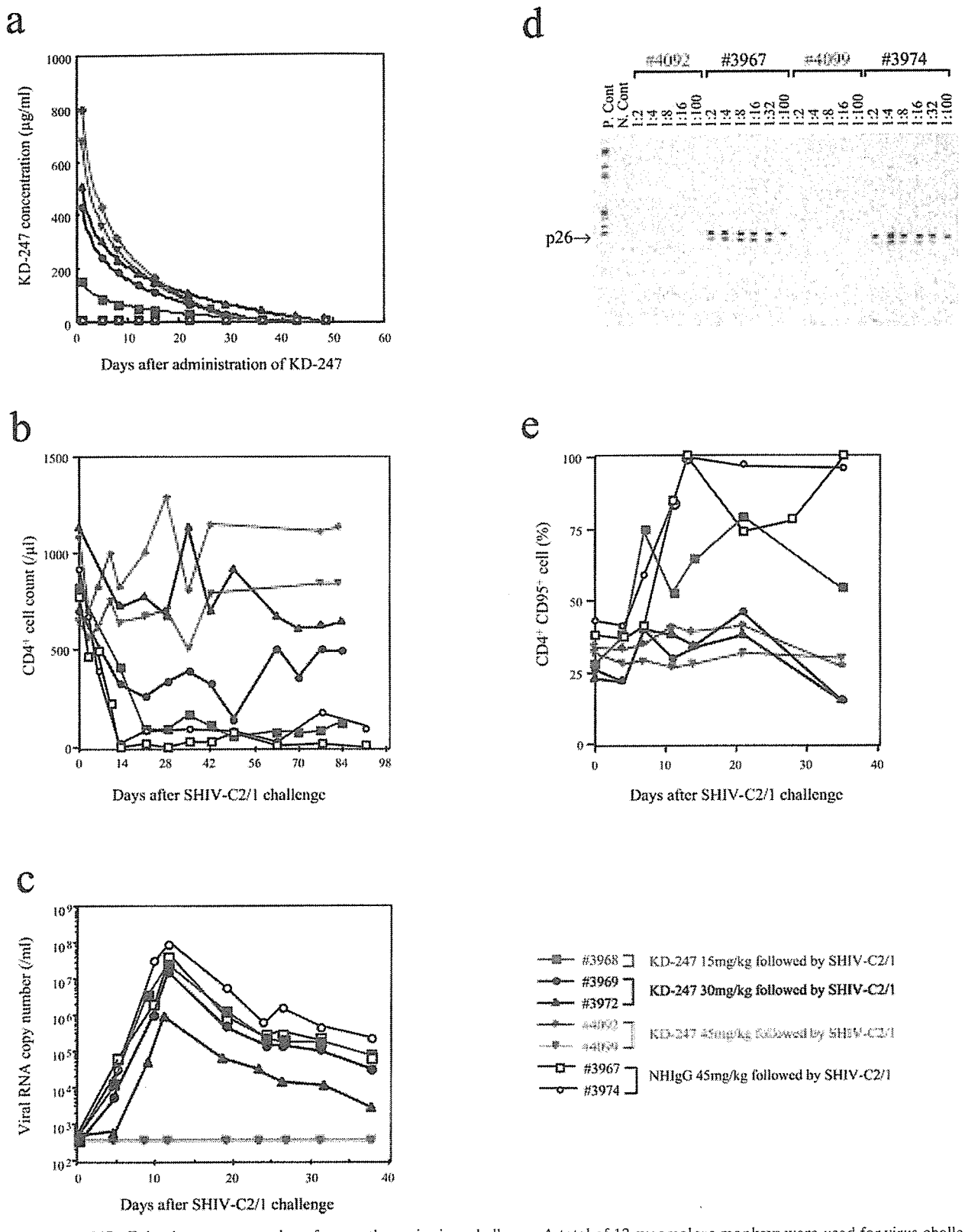

FIG. 1. KD-247 efficiently protects monkeys from pathogenic virus challenge. A total of 12 cynomolgus monkeys were used for virus challenge studies with SHIV C2/1. In the first group, five monkeys were intravenously inoculated with various doses of KD-247, followed by 20 $TCID_{50}$s of SHIV C2/1 challenge 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.

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JOURNAL OF VIROLOGY, Nov. 2006, p. 10683–10691 Vol. 80, No. 21
0022-538X/06/$08.00+0 doi:10.1128/JVI.01009-06
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De Novo Human T-Cell Leukemia Virus Type 1 Infection of Human Lymphocytes in NOD-SCID, Common γ-Chain Knockout Mice▽

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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

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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-*yc*^{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′-GAAGACTGTTTGCCCACCACCC-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′-CCCGGCGCTGCTCTCATCCCGG-3′ and 5′-GGCCGAACATA GTCCCCCAGAG-3′ for *tax* and 5′-GCAGGGGGGGAGCCAAAAGGG-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) (50), 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 10^5 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-I 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-I 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-I, 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-I infection.

In order to confirm HTLV-I 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

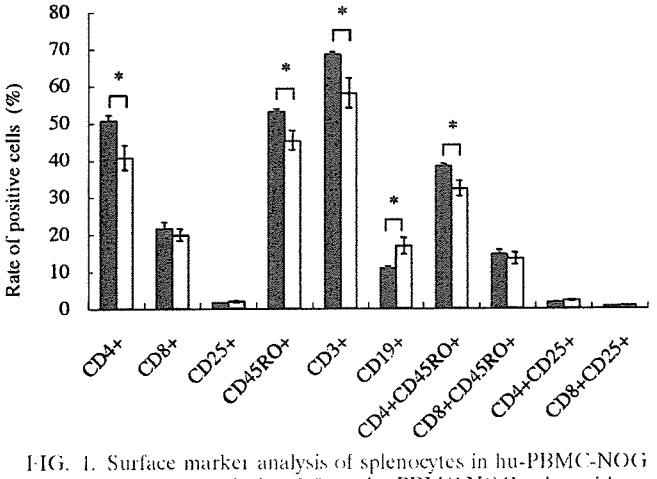

FIG. 1. Surface marker analysis of splenocytes in hu-PBMC-NOG mice. Splenocytes were isolated from hu-PBMC-NOG mice with or without HTLV-1 infection, and their surface markers were analyzed by flow cytometry. Splenocytes were recovered at 2 weeks p.i. The percentages of cells positive for various surface 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)

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).

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).

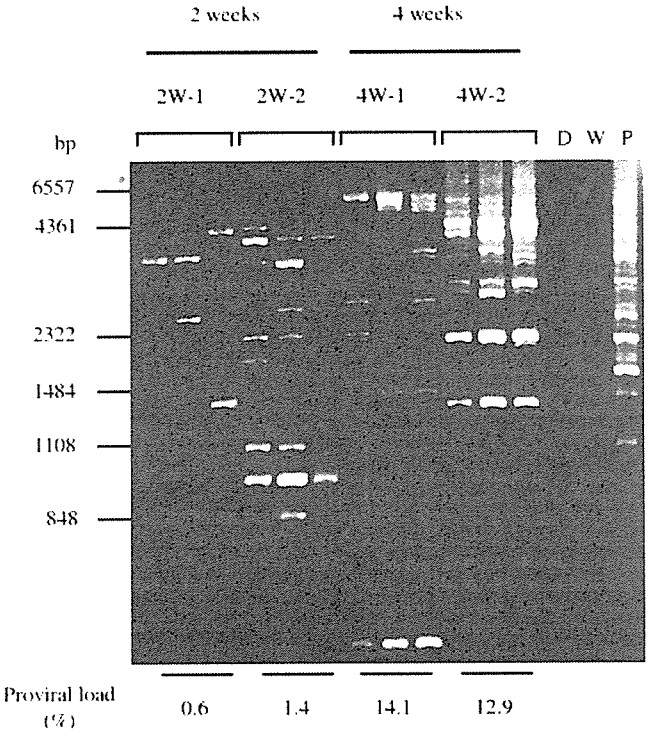

FIG. 2. Polyclonal proliferation of HTLV-1-infected cells in the spleens of hu-PBMC-NOG mice (2W-1, 2W-2, 4W-1, and 4W-2). Genomic DNA was isolated from recovered splenocytes and analyzed by IL-PCR as described in Materials and Methods. IL-PCR was performed in triplicate for each DNA sample. Genomic 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.

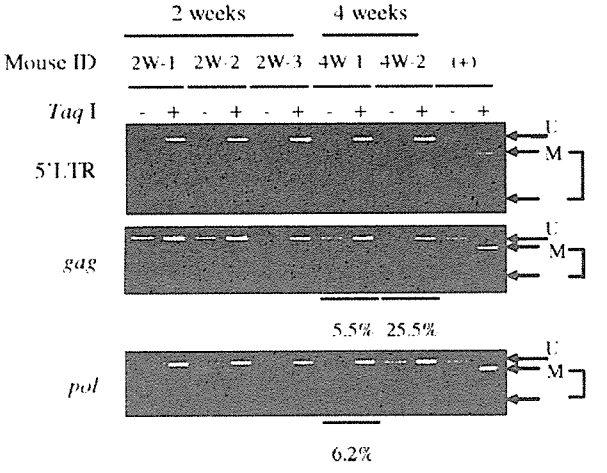

FIG. 3. DNA methylation of HTLV-1 provirus. hu-PBMC-NOG mice were sacrificed 2 or 4 weeks after inoculation of MT-2 cells, and DNA methylation in the 5′ LTR, *gag*, and *pol* regions was studied by a COBRA assay. (+), positive control; U, intact fragment (unmethylated CpG); M, digested fragments (methylated 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-

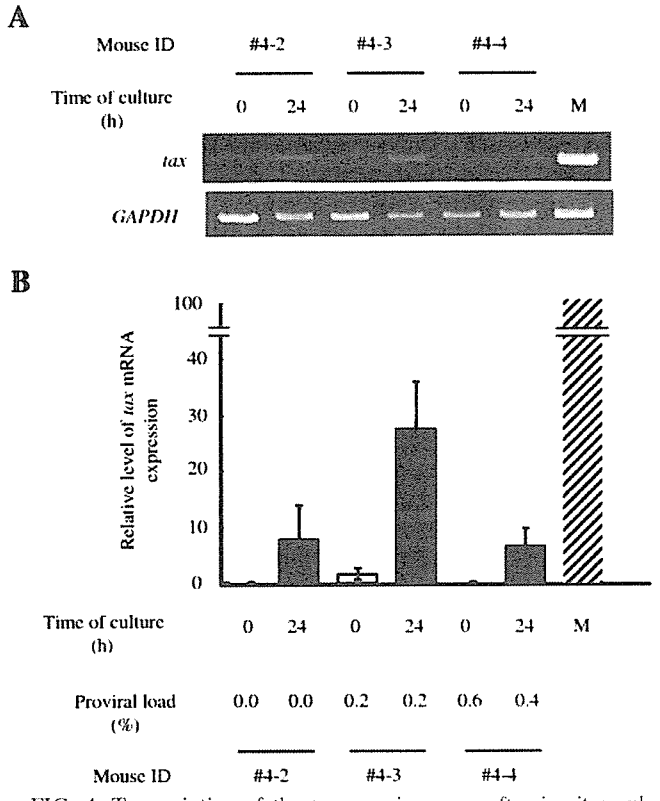

FIG. 4. Transcription of the *tax* gene increases after in vitro culture. Splenocytes of hu-PBMC-NOG mice inoculated with 10^4 MT-2 cells were recovered 2 weeks after infection. Transcription of the *tax* gene was quantified by semiquantitative PCR (A) or real-time PCR (B) at recovery and after 24 h 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.

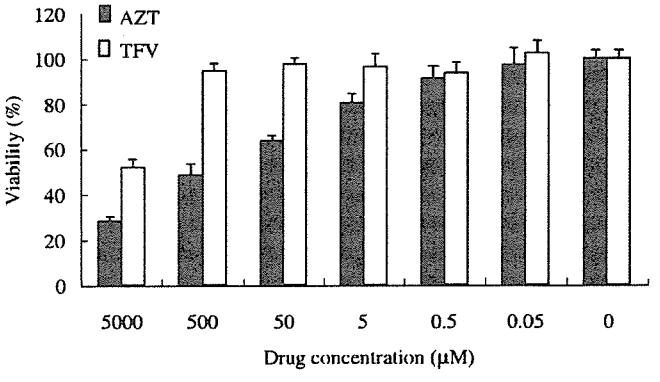

FIG. 5. Cytotoxic effects of tenofovir (TFV) and AZT in vitro. Human PBMC were stimulated with PHA for 3 days. Cells were then cultured in medium alone or medium containing the specified concentration of the indicated drug for another three days, at a density of 10^5 cells/well, in a 96-well plate. Viability 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.

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HIV Encephalopathy

JMAJ 49(5・6): 212–218, 2006

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Abstract

HIV encephalopathy is one of the most complex viral diseases. HIV-infection is mainly restricted to macrophages and microglia in HIV-infected brains, although HIV-induced damage extends to neurons and oligodendrocytes. Accumulating evidences suggest that HIV-encoded factors and other host factors are involved in the development of this disease: however, the precise mechanism remains unclear. In order to investigate this mechanism, we developed an HIV-1-infected human cell-transplanted mouse model (hu-PBMC-NOD-SCID mouse) and a coculture system with HIV-1-infected macrophages and murine or rat brain cells. Using these models, we have successfully determined that certain host factors are involved in the neuronal damage. Additionally, we are developing a screening system to identify the host factors that provide protection against HIV-1-induced encephalopathy. Our study will contibute to the development of a new therapeutic strategy for HIV encephalopathy and other CNS diseases.

Key words HIV encephalopathy, Macrophage, hu-PBMC-NOD-SCID mouse, A lentiviral screening system, Host factors, CNS diseases

Introduction

The human immunodeficiency virus type 1 (HIV-1) causes acquired immunodeficiency syndrome (AIDS) by the destruction of the immune system.[1–3] However, the target tissues are not restricted to those of the immune system. This virus invades the central nervous system (CNS) and induces a neurological disease called HIV encephalopathy. Most cases of this disease are diagnosed several years after the primary infection, and by then, the number of $CD4^+$ cells in the peripheral blood is significantly lower. The clinical symptoms of this disease include cognitive, behavioral, and motor dysfunction: these symptoms are characteristically found in subcortical dementia and commonly develop over a period of few months. In the early stage, forgetfulness and reduced concentration are frequently encountered and these are typically followed by short-term memory loss, carelessness and mental slowing. Motor disturbance in the form of leg weakness is sometimes observed at this stage. These symptoms often occur along with behavioral symptoms such as personality changes. Ataxia, tremor, pyramidal sign, and paresis are also found. On disease progression, the patient shows behavioral changes such as social withdrawal, apathy, akinetic and mute state.

New Problems After the Introduction of HAART

In 2004, approximately 40 million people worldwide were estimated to be already infected with HIV. However, a very effective anti-viral therapy called highly active antiretroviral therapy (HAART) that comprises a combination of HIV reverse transcriptase and protease inhibitors has been available since around 1997. Following the availability of this therapy, the number of deaths

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