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Animal model for the therapy of acquired immunodeficiency syndrome with reverse transcriptase inhibitors

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ABSTRACT **The reverse transcriptase (RT) of the human immunodeficiency virus type 1 (HIV-1) is the major target for antiretroviral therapy of the acquired immunodeficiency syndrome (AIDS). While some inhibitors exhibit activity against most retroviral RTs, others are specific for the HIV-1 enzyme. To develop an animal model for the therapy of the HIV-1 infection with RT inhibitors, the RT of the simian immunodeficiency virus (SIV) was replaced by the RT of HIV-1. Macaques infected with this SIV/HIV-1 hybrid virus developed AIDS-like symptoms and pathology. The HIV-1-specific RT inhibitor LY300046·HCl, but not zidovudine [3′-azido-3′-deoxythymidine (AZT)] delayed the appearance of plasma antigenemia in macaques infected with a high dose of the chimeric virus. Infection of macaques with the chimeric virus seems to be a valuable model to study the *in vivo* efficacy of new RT inhibitors, the emergence and reversal of drug resistance, the therapy of infections with drug-resistant viruses, and the efficacy of combination therapy.**

All drugs approved for antiretroviral therapy of the human immunodeficiency virus type 1 (HIV-1) infection inhibit the reverse transcriptase (RT) of HIV-1. Numerous other RT inhibitors have been described (1), and some of them are in clinical trials. Although these drugs strongly inhibit viral replication *in vitro*, the long-term benefits of these drugs on clinical progression of disease are uncertain (2). After prolonged therapy, drug-resistant viruses emerge because of point mutations in the polymerase gene that give rise to amino acid changes in RT (3–7). The limited efficacy of existing therapies and the development of drug-resistant viruses stress the need for better drugs or therapeutic regimens. The preclinical evaluation of the efficacy of drugs is mainly based on *in vitro* assays. The strong antiviral effects *in vitro* do not correlate well with the efficacies observed *in vivo*. Infection of macaques with simian immunodeficiency virus (SIV) and HIV type 2 (HIV-2) seems to offer better models for predicting antiviral effects of some drugs in humans (8–15). Since the infection of macaques with SIV closely mimics HIV-1 infection in humans and since SIV-infected monkeys develop a disease very similar to the acquired immunodeficiency syndrome (AIDS) (16–19), it is a model in which the effect of antiretroviral therapy on disease progression could be studied. However, the infection of macaques with SIV as a therapeutic model has some limitations. Although the RTs of SIV and HIV-1 are approximately 60% homologous, they differ in their susceptibility to nonnucleoside RT inhibitors (1). Furthermore, it is unlikely that development of drug resistance involves the same amino acid changes in both viruses. The other most commonly used animal model, the infection of humanized severe combined immunodeficient mice (SCID) with HIV-1 (20–23), has some theoretical limitations that led to doubts about the predictive value for the clinical efficacy of drugs. HIV-1 infection of humanized SCID mice did not induce disease. HIV-1 infection led to the destruction of susceptible graft cells within a few weeks (24). In addition, the viral load and the number of quasi-species must be orders of magnitude less than in humans. Therefore, it is difficult to assess the development of drug resistance in this model. Since development of drug resistance seems to be a major reason for the failure of existing therapies, an animal model that can predict the clinical benefits and the timing of emergence of drug resistance is urgently needed. In an attempt to overcome some of the limitations of the SIV model, macaques were infected with a SIV/HIV-1 hybrid virus expressing HIV-1 RT (RT-SHIV).

MATERIALS AND METHODS

Construction of RT-SHIV. The SIVmac239 provirus (25) was propagated in *Escherichia coli* by using two plasmids, one containing the 5′ half (p239ESp5′) and one containing the 3′ half (p239SpE3′/nef-open) (26). To construct RT-SHIV, the RT gene of SIVmac239 was first deleted from the 5′ half. This was done by ligating a PCR-generated fragment spanning all SIV sequences 5′ of the RT gene with a PCR-generated fragment spanning the sequence 3′ of the RT gene up to the unique *Sph* I site. The following primers were used: 5′ flank, 5′-TCGAGAATTCCTTGCAGGTTTCTGGAAGGGA-3′; anti-prot, 5′-GCAACCCGGGAAAATTTAGAGACATCCCCAG-3′; int, 5′-ATTACCCGGGATTAGAAAAGTTCTCTTCTTGGA-3′; anti-tat, 5′-TCGAGCGGCCGCATGCTTCTAGAGGGCGGTATAG-3′. The fragments were joined by the *Xma* I sites in the primers anti-prot and int. A 190-base-pair *Bsp*HI–*Bst*XI fragment containing the junction between protease and integrase DNAs was sequenced and cloned back into the same sites of p239ESp5′, generating p239dRT. The PCR-amplified RT DNA of the HIV-1 IIIB clone HXBc2 (27) (primers: RT, 5′-ATTAGCCCTATTGAGACTGTACCA-3′; anti-RT, 5′-AGCACTGACTAATTTATCTACTTG-3′) was cloned into the *Sma* I site generated at the junction of protease and integrase DNAs in p239dRT, resulting in RT-SHIV5′. The integrity of the junctions between protease and RT DNAs and RT and integrase DNAs was confirmed by sequence analysis.

Cell Culture Experiments. The plasmids containing the 5′ halves of the viral genomes RT-SHIV5′ and p239ESp5′ were

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Abbreviations: HIV-1, human immunodeficiency virus type 1; SIV, simian immunodeficiency virus; RT, reverse transcriptase; RT-SHIV, SIV/HIV-1 hybrid virus expressing HIV-1 RT; PBMC, peripheral blood mononuclear cells; AZT, 3′-azido-3′-deoxythymidine.

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digested with *Sph* I and *Eco*RI and ligated to the *Eco*RI- and *Sph* I-digested p239SpE3′/nef-open plasmid. Five micrograms of ligated SIVmac239 or RT-SHIV plasmid clones was transfected into CEMx174 cells (37) by the DEAE-dextran method, and virus stocks from the supernatant of these cells were prepared by passing the cleared cell culture supernatant through a 0.22-μm filter prior to storage at −70°C (28). Peripheral blood mononuclear cells (PBMC) of rhesus monkeys were infected with 2500 units of RT activity of the respective viruses as described (28). The RT activity in the culture medium of the infected or uninfected PBMC was determined according to standard procedures (29). The tissue culture 50% infectious dose ($TCID_{50}$) of RT-SHIV was determined as described (30) in the presence of 0, 50, 100, and 200 nM Nevirapine (BI-RG-587) (38). A RT-SHIV stock was prepared in rhesus monkey PBMC as described (28). The stock was titered on CEMx174 cells (30). The minimal number of PBMC required for virus isolation in cocultures with CEMx174 cells was determined as a measure of cell-associated viral titers. Virus replication was verified by measuring the RT activity in culture supernatants (31) or by detecting viral antigens with a polyvalent antigen capture assay (Organon).

RT Assays. Viral particles from the cleared supernatant of SIV-, RT-SHIV-, or HIV-1-infected CEMx174 cells were pelleted by ultracentrifugation at 27,000 rpm for 90 min in a SW Ti45 rotor. Pellets were resuspended in 0.5 M KCl/1 mM dithiothreitol/0.1% Triton X-100/5 mM Tris, pH 7.8. *In vitro* RT assays were performed in the presence of the indicated concentrations of Nevirapine for 45 min at 37°C in 50 mM Tris (pH 7.8) containing 0.067% Triton X-100, 10 mM $MgCl_2$, 83 mM KCl, 0.16 mM dithiothreitol, 1 μM dGTP, 1.5 nM [α-^{32}P]dGTP (3000 Ci/mmol), and 0.5 A_{260} units of poly(rC)·p$(dG)_{12-18}$ (Pharmacia) per ml.

Immunoprecipitations. Approximately 2×10^6 uninfected, SIVmac239-, RT-SHIV-, or HIV-1 (HXBc2)-infected CEMx174 cells were metabolically labeled overnight with 600 μCi (1 μCi = 37 kBq) of [^{35}S]methionine and [^{35}S]cysteine. Viral particles from the cleared culture supernatant were pelleted through a 20% sucrose cushion at 27,000 rpm for 90 min in a SW Ti45 rotor. Immunoprecipitations were performed with an HIV-1 patient serum or a monoclonal antibody directed against HIV-1 RT (catalog no. 9001, American Biotechnologies, Cambridge, MA) and protein A-coupled Sepharose beads. The precipitated material was analyzed on an SDS/11.5% polyacrylamide gel.

Infection of Macaques. Rhesus monkeys were housed at the German Primate Center in Göttingen. Handling of the monkeys and collection of specimens were performed according to institutional guidelines as described (32). Two rhesus monkeys of Indian origin (seronegative for SIV, D-type retroviruses, and simian T-cell lymphotropic virus type 1) were infected intravenously with 3000 $TCID_{50}$ of RT-SHIV. The amount of p27 capsid antigen in the plasma was determined by using an antigen capture assay (Coulter). Cynomolgus monkeys were housed at the Center for Microbiology and Tumorbiology, Karolinska Institute, Stockholm. Housing and handling of the macaques was as described earlier (8). Twelve cynomolgus monkeys were infected intravenously with 200 $TCID_{50}$ of the RT-SHIV stock. Four control animals were not treated, four macaques received 15 mg of 3′-azido-2′,3′-dideoxythymidine (AZT; zidovudine; Retrovir) per kg of body weight three times daily subcutaneously, and four macaques received 5 mg of LY300046·HCl per kg of body weight (dissolved in saline) three times daily subcutaneously; LY300046·HCl (Trovirdine), supplied by Medivir, is an Eli Lilly code name for *N*-[2-(2-pyridyl)ethyl]-*N*′-[2-(5-bromo)pyridyl]thiourea hydrochloride. The first and second dose were given 8 hr and 15 min before virus inoculation, respectively. Treatment was continued every 8 hr for 5 days. Sera were collected at intervals, and the p27 antigen and anti-SIV antibodies were determined as described (8, 33).

Determination of Immunological Parameters. The humoral immune response of infected monkeys against viral antigens was determined by ELISA using whole SIV as antigen as described (34). The CD4/CD8 ratio in the PBMC of infected macaques was determined in fluorescence-activated cell-sorting (FACS) analyses using a fluorescein isothiocyanate (FITC)-labeled anti-CD4 antibody (OKT4, Ortho Diagnostics) and a FITC-labeled anti-CD8 antibody (B9.11, Dianova, Hamburg, Germany).

RESULTS

Construction and Characterization of RT-SHIV. To develop an animal model for the therapy of AIDS with RT inhibitors, the RT of SIVmac239 (25) was replaced by the RT of the HxB2 clone of HIV-1 (27) (Fig. 1 *Upper*). To determine whether this chimeric virus, designated RT-SHIV, was replication competent, CEMx174 cells were transfected with RT-SHIV DNA. Cytopathic effects and RT activity could be detected 3 weeks after transfection (data not shown). To compare the replication kinetics of SIVmac239 with RT-SHIV, PBMC from rhesus monkeys were infected with the supernatant of SIVmac239- or RT-SHIV-transfected CEMx174 cells, which had been normalized for RT activity. RT-SHIV replicated to high titers in rhesus monkey PBMC, although replication was delayed compared with that of SIVmac239 (Fig. 1 *Lower*). All proteins of RT-SHIV should be of SIVmac239 origin, with the exception of the RT, which should be derived from HIV-1. To determine whether RT-SHIV expresses the predicted proteins, virus particles from the supernatant of metabolically labeled SIV-, HIV-1-, or RT-SHIV-infected cells were immunoprecipitated with an HIV-1 patient serum, which also cross-reacts with SIV Gag and Pol proteins. The immunoprecipitated material was analyzed by SDS/polyacrylamide gel elec-

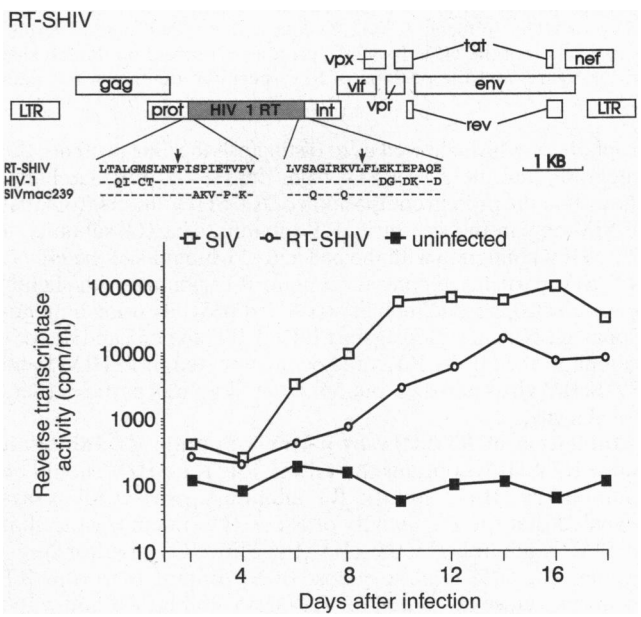

FIG. 1. Construction and *in vitro* characterization of RT-SHIV. (*Upper*) Genome organization of RT-SHIV. Genes derived from SIVmac239 are marked by white boxes; the HIV-1-specific RT gene sequence is shaded. The corresponding amino acid sequence of RT-SHIV at the junction of HIV-1 and SIVmac239 sequences is shown in comparison to the amino acid sequences of the parental viruses. Vertical arrows denote the presumed protease cleavage sites. (*Lower*) Replication of SIVmac239 (SIV) and RT-SHIV in PBMC of rhesus monkeys.

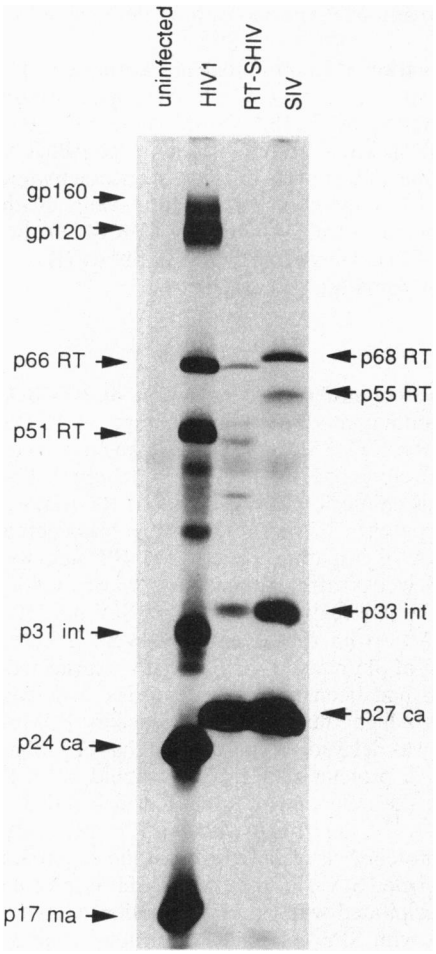

FIG. 2. Protein composition of RT-SHIV. Immunoprecipitations of virus particles from the supernatant of uninfected, RT-SHIV-, SIV-, or HIV-1-infected CEMx174 cells with a HIV-1 patient serum. The position of the HIV-1-specific proteins is marked on the left side of the figure, and the position of SIV-specific proteins, on the right side.

trophoresis, which allowed us to distinguish the Gag proteins, the integrase, and the RT of SIV from the HIV-1 proteins. Fig. 2 shows that the protein composition of RT-SHIV differs from that of SIV only in the size of its RT subunits. The RT subunits of RT-SHIV comigrate with the p65 and p51 subunits of the HIV-1 RT. SIV particles in contrast contain RT subunits with slightly lower electrophoretic mobility (p68 and p55). By using a monoclonal antibody directed against HIV-1 RT, the p65 and the p51 subunit of the HIV-1 RT could be precipitated from HIV-1 and RT-SHIV virus particles, but not from SIV virus particles (data not shown).

Inhibition of RT-SHIV by a HIV-1-Specific RT Inhibitor. Since RT-SHIV contains the HIV-1 RT, RT-SHIV should be inhibited by HIV-1-specific RT inhibitors. *In vitro* RT assays revealed that the RT activity of RT-SHIV, in contrast to that of SIV, is inhibited by the HIV-1-specific RT inhibitor Nevirapine. The 50% inhibitory dose of Nevirapine in *in vitro* RT assays was close to 0.5 μM for RT-SHIV and HIV-1 and >100 μM for SIV (Fig. 3). The $TCID_{50}$ of a RT-SHIV stock was reduced by 58% in 50 nM Nevirapine, by 81% in 100 nM Nevirapine, and by 88% in 200 nM Nevirapine. The replication of SIVmac239 was not inhibited significantly by Nevirapine even at 2.5 mM concentration (data not shown). This demonstrates that both RT activity and virus replication of RT-SHIV could be inhibited by a HIV-1-specific RT inhibitor.

Infection of Rhesus Monkeys with RT-SHIV. To characterize the course of RT-SHIV infection in macaques, two rhesus

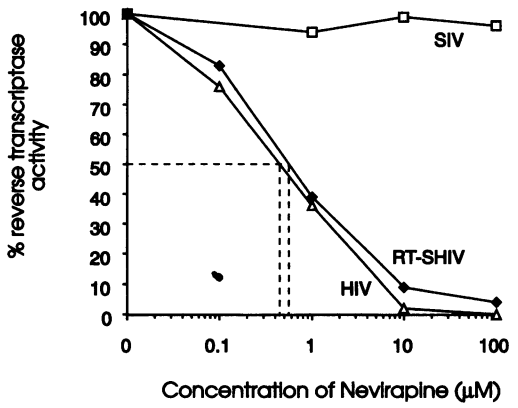

FIG. 3. Inhibition of RT activity by Nevirapine. *In vitro* RT assays were performed with precipitated HIV-1, RT-SHIV, or SIV in the presence of the indicated concentrations of Nevirapine. The background activity was subtracted from the mean of triplicates, and the values are expressed as the percentage of the RT activity in the absence of Nevirapine.

monkeys were inoculated with 3000 $TCID_{50}$ of a RT-SHIV stock. Infection was evident by SIV p27 serum antigenemia 2 weeks after inoculation and by increasing antibody titers against SIV (Table 1). A high viral load in PBMC was detected throughout the observation period (Table 1). Four weeks after inoculation, both monkeys developed persistently enlarged, palpable lymph nodes. Six months after infection, the CD4/CD8 ratio in the PBMC had declined to 0.5 and 0.7 (Table 1). At that time, one macaque (1833) had to be euthanized because of severe ataxia. The pathological examination revealed a purulent encephalitis and follicular involution in the spleen and the lymph nodes. Although we had no evidence of a contamination of the RT-SHIV-infected macaques with SIV, we wanted to make sure that the observed symptoms were due to RT-SHIV infection. Therefore, sequence analyses of isolates recovered from both macaques 6 months after infection were performed. While there were a number of point mutations in the HIV-1 RT gene, no SIV RT genes were detected, thereby excluding a contaminating SIV infection (data not shown). The reisolates recovered at that time were still sensitive to Nevirapine (data not shown). One year after infection, the second macaque is still alive with a persistent lymphadenopathy and a low CD4/CD8 ratio.

Determination of the *in Vivo* Efficacy of Compounds. For a more widespread use of the RT-SHIV/macaque model in drug development, it would be important to have an assay that allows the rapid testing of a large number of compounds and dosing regimens. Therefore, we analyzed the effect of a short-term drug treatment on viral replication. Four macaques were treated with the standard antiretroviral drug AZT and four macaques were treated with the potent HIV-1-specific RT inhibitor LY300046·HCl. LY300046·HCl is the first member of the PETT (phenylethylthioureathiazole) nonnucleoside RT inhibitors that is being evaluated in a phase I clinical trial (C. Ahgren, B.Ö., and collaborators, unpublished data). As a control, four untreated macaques were included. Treatment was initiated 8 hr before virus inoculation and continued every 8 hr for 5 days. In LY300046·HCl-treated macaques, there was a significant delay in the appearance of SIV-p27 antigenemia (Fig. 4 *Upper*) and in the appearance of anti-SIV antibodies (Fig. 4 *Lower*). This was not observed in AZT-treated macaques.

DISCUSSION

Inoculation of rhesus monkeys with RT-SHIV initiated an efficient infection. The progressing lymphadenopathy with

Table 1. Follow-up of rhesus monkeys inoculated with RT-SHIV

Weeks after infection	Anti-SIV ELISA titer $\times 10^{-2}$		p27 antigenemia, ng/ml		Minimal number of PBMC required for virus isolation		CD4/CD8	
	1832	1833	1832	1833	1832	1833	1832	1833
2	<1	<1	2.2	0.7	800	1,700	ND	ND
4	16	32	Neg	Neg	1,700	1,700	1	1.1
8	512	256	Neg	Neg	400	3,500	0.8	1.1
12	512	1024	ND	ND	<3,500*	<15,000*	1.6	1.5
16	1024	1024	ND	ND	800	1,700	0.9	1.4
20	2048	1024	Neg	Neg	3,500	1,700	0.8	1.1
24	ND	ND	ND	Neg	ND	400	0.7	0.5
26	ND	D	ND	D	3,500	D	0.8	D
32	ND		0.4		3,500		0.5	
40	ND		ND		7,000		0.4	
44	ND		ND		15,000		0.5	
52	ND		ND		1,700		0.3	

*, Culture had to be discarded before the endpoints of the virus isolation were reached. ND, not determined; D, dead; Neg, antigenemia not detectable.

follicular involution and the decline in the CD4/CD8 ratios show that RT-SHIV can induce AIDS-like symptoms and pathology. The purulent encephalitis observed upon autopsy in one of the macaques indicated a secondary bacterial infection. Such a severe incidental infection of the central nervous system of healthy macaques is rather unlikely. In addition, the low CD4/CD8 ratio and the absence of a SIV antigen-specific T-cell proliferation (U. Dittmer and K.Ü., unpublished observation) strongly indicated that this macaque developed simian AIDS. Cynomolgus monkeys could also be infected with RT-SHIV and responded with the same acute symptoms. The follow-up of more macaques is necessary to evaluate fully the pathogenic potential of RT-SHIV.

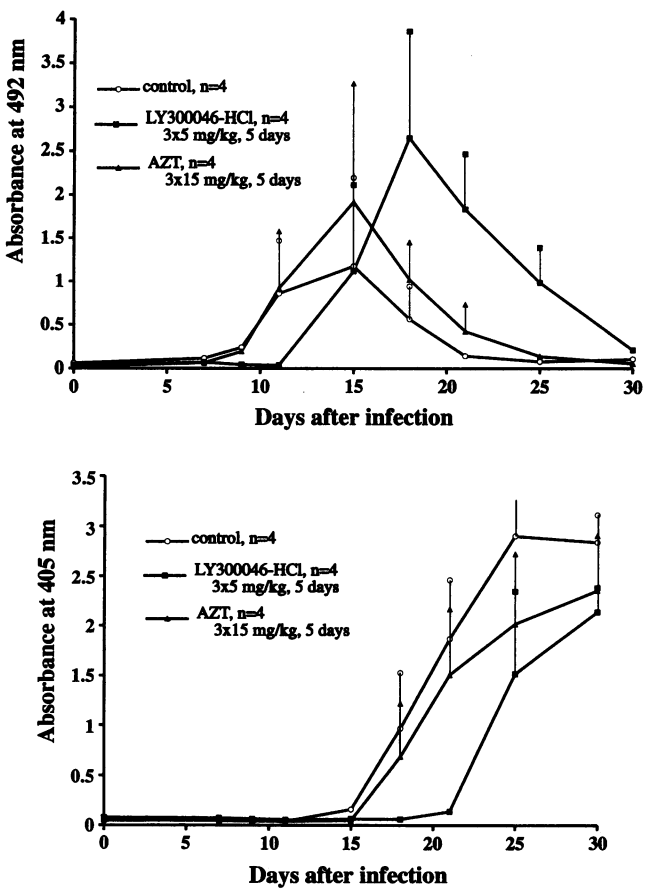

FIG. 4. Effect of AZT and LY300046·HCl on the appearance of SIV p27 antigen (*Upper*) and anti-SIV antibodies (*Lower*) in sera of RT-SHIV-infected macaques. The values shown represent the mean of each of the control and experimental groups. For mean absorbance values more than 0.25, the values of the mean plus the standard deviation are also shown linked to the mean values by a vertical line.

Although the genome organization of HIV-1, HIV-2, and SIV is similar, they differ in their host tropism. While HIV-2 and SIV can infect humans and different monkey species, productive HIV-1 infection is mainly restricted to humans and chimpanzees. By using HIV-1/SIV hybrid viruses, it was shown that the inability of HIV-1 to replicate in rhesus monkey PBMC was not due to the HIV-1 *env, tat, rev,* or *vpu* genes (28, 35). The infection of macaques with RT-SHIV indicated that the RT of HIV-1 does not determine the restricted host tropism of HIV-1 either.

Treating cynomolgus monkeys for 5 days during the acute phase of RT-SHIV infection with AZT did not lead to a delay in the appearance of antigenemia. Using the same dosage regimen of AZT for the treatment of SIV-infected cynomolgus monkeys, we had observed a delay in the appearance of antigenemia when the virus inoculum was <100 monkey 50% infectious doses (MID_{50}) (8). The ratio of the monkey infectious dose of a second RT-SHIV stock to its tissue culture infectious dose was approximately 5 (B.Ö., unpublished observation). From the tissue culture infectious dose of our first RT-SHIV stock, we would estimate that the macaques in the therapy experiment were inoculated with approximately 1000 MID_{50}. Therefore, the delay observed with a 5-day treatment of LY300046·HCl demonstrates a potent antiretroviral activity *in vivo*. The increased antigenemia levels observed in LY300046·HCl-treated macaques are probably due to the fact that no samples were drawn from the control group between days 11 and 15. Since half of the untreated macaques had rather low peak antigen levels on day 11, while the other half had low peak antigen levels on day 15, antigen values in the control group probably peak between days 11 and 15. In contrast, all four LY300046·HCl-treated macaques showed peak antigen levels on day 18. Long-term treatment of RT-SHIV-infected macaques in an acute and chronic infection will be necessary to evaluate the development of drug resistance and survival benefits. However, in view of the good prediction of antiviral effects in SIV- and HIV-2-infected monkeys, it seems likely that effects in the RT-SHIV model will predict antiviral effects in patients better than cell culture experiments do.

Since the emergence of drug resistance is a major reason for the failure of existing therapies, it would be important to have a model in which drug resistance could be studied. Passaging

of RT-SHIV in increasing concentration of Nevirapine led to the emergence of viral variants containing a valine-to-alanine mutation at position 106 of RT (K.Ü., unpublished observation), which is also found in Nevirapine-resistant HIV-1 (36). Introduction of four mutations associated with resistance of HIV-1 to AZT (7) into the RT gene of RT-SHIV conferred AZT resistance (K.Ü. and H. Linhardt, unpublished observation). Therefore, this animal model should allow study of the emergence and reversal of drug resistance and the therapy of drug-resistant viruses. The model makes possible a direct comparison of the *in vivo* efficacies of compounds directed against the HIV-1 RT and should help to focus clinical trials on the most promising drugs, combination therapies, and treatment regimens.

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