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Effective Suppression of Human Immunodeficiency Virus Type 1 through a Combination of Short- or Long-Hairpin RNAs Targeting Essential Sequences for Retroviral Integration

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Small interfering RNA (siRNA) could provide a new therapeutic approach to treating human immunodeficiency virus type 1 (HIV-1) infection. For long-term suppression of HIV-1, emergence of siRNA escape variants must be controlled. Here, we constructed lentiviral vectors encoding short-hairpin RNAs (shRNA) corresponding to conserved target sequences within the integrase (*int*) and the attachment site (*att*) genes, both of which are essential for HIV-1 integration. Compared to shRNA targeting of the HIV-1 transcription factor *tat* (shTat), shRNA against *int* (shIN) or the U3 region of *att* (shU3) showed a more potent inhibitory effect on HIV-1 replication in human CD4$^+$ T cells. Infection with a high dose of HIV-1 resulted in the emergence of escape mutants during long-term culture. Of note, limited genetic variation was observed in the viruses resistant to shIN. A combination of shINs against wild-type and escape mutant sequences had a negative effect on their antiviral activities, indicating a potentially detrimental effect when administering multiple shRNA targeting the same region to combat HIV-1 variants. The combination of shIN and shU3 *att* exhibited the strongest anti-HIV-1 activity, as seen by complete abrogation of viral DNA synthesis and viral integration. In addition, a modified long-hairpin RNA spanning the 50 nucleotides in the shIN target region effectively suppressed wild-type and shIN-resistant mutant HIV-1. These results suggest that targeting of incoming viral RNA before proviral DNA formation occurs through the use of nonoverlapping multiple siRNAs is a potent approach to achieving sustained, efficient suppression of highly mutable viruses, such as HIV-1.

Gene targeting in mammalian cells through the use of short-hairpin RNAs (shRNAs) has been advanced by the development of vector systems for efficient delivery and stable expression of shRNA sequences (4, 7, 22, 30). Upon delivery into cells, shRNAs are converted into short double-stranded RNAs, termed small interfering RNAs (siRNAs), that mediate a sequence-specific RNA degradation process termed RNA interference (RNAi) (12, 14, 42). Antiviral therapy based on siRNA has been proposed as a new method for intracellular immunization against human immunodeficiency virus type 1 (HIV-1) (16, 31, 32) and hepatitis C virus (HCV) (34). When viral genes are targeted, viruses can escape from RNAi-mediated inhibition due to their high mutation rate (6, 11, 39). An alternative approach that shows promise is the use of siRNAs targeting cellular genes essential for virus replication. In the case of HIV-1, siRNAs against the cell surface CD4 receptor (31) or CXCR4 and CCR5 coreceptors for HIV-1 entry conferred viral resistance (2, 3, 33). However, CD4 and CXCR4 are essential for T-cell development and proper immunologic function. In addition, although CCR5 might be nonessential for normal function (23), not all HIV-1 strains require CCR5. Downregulation of an essential cellular coreceptor could potentially result in the emergence of HIV-1 variants that use another coreceptor(s) for viral entry into the cell.

To achieve long-term control of viral replication by siRNA and prevent the emergence of escape variants, it is important to target highly conserved and/or essential HIV-1 sequences. For example, many sites in the *cis*-regulatory regions, as well as the protein-coding regions, of HIV-1 have been examined as potential targets for siRNA. These regions include the primer-binding site, the polypurine tract, the long terminal repeat, and the *gag*, *pol*, *env*, *tat*, *rev*, *vif*, and *nef* genes (6, 10, 11, 16, 18, 21, 30, 39). The degree to which siRNAs inhibited HIV-1 replication and the underlying mechanisms varied considerably, depending on the target sequence (10, 11). For example, RNAi-resistant HIV-1 variants can emerge not only through mutations in the siRNA target sequence but also through mutations that alter the local RNA structure (39). These results emphasize the need for empirical studies to determine effective siRNA target sites within the HIV-1 genome.

In the present study, we selected several sequences for lentivirus-mediated shRNA expression based on a preliminary screening of HIV-1 RNAi target sites using synthetic siRNA duplexes. These sequences mapped within the integrase (IN) gene (*int*) and the attachment site (*att*), which are essential for HIV-1 integration. We evaluated the anti-HIV-1 activity of these expressed shRNAs using a highly susceptible CD4$^+$ T-cell line. Genetic analysis of HIV-1 escape mutants that emerged after treatment with combinations of shRNAs revealed that two or more shRNAs targeting different essential sequences had the strongest impact on

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antiviral activity. The results also suggest that shRNAs or long-hairpin RNA (lhRNA) that targets incoming viral RNA before proviral DNA formation is more efficient at mediating RNAi antiviral therapy.

MATERIALS AND METHODS

Construction of plasmids. A series of small-hairpin-RNA-expression vectors were constructed using pGEM-H1 and pCS-H1 vectors described previously (30). Sense (S) and antisense (AS) sequences for shRNA were as follows: shTat-S, 5′-GAT CCC CTG CTT GTA CCA ATT GCT ATT CAA GAG ATA GCA ATT GGT ACA AGC AGT TTT TGG AAA G-3′; shTat-AS, 5′-TCG ACT TTC CAA AAA CTG CTT GTA CCA ATT GCT ATC TCT TGA ATA GCA ATT GGT ACA AGC AGG G-3′; shIN-S, 5′-GAT CCC GGA GAG CAA TGG CTA GTG ATT CAA GAG ATC ACT AGC CAT TGC TCT CCT TTT TGG AAA G-3′; shIN-AS, 5′-TCG ACT TTC CAA AAA GGA GAG CAA TGG CTA GTG ATC TCT TGA ATC ACT AGC CAT TGC TCT CCG G-3′; shU3-S, 5′-GAT CCC GAC TGG AAG GGC TAA TTC ATT CAA GAG ATG AAT TAG CCC TTC CAG TCT TTT TGG AAA G-3′; shU3-AS, 5′-TCG ACT TTC CAA AAA GAC TGG AAG GGC TAA TTC ATC TCT TGA ATG AAT TAG CCC TTC CAG TCG G-3′; shIN-G4288A-S, 5′-GAT CCC GGA AAG CAA TGG CTA GTG ATT CAA GAG ATC ACT AGC CAT TGC TTT CCT TTT TGG AAA G-3′; shIN-G4288A-AS, 5′-TCG ACT TTC CAA AAA GGA AAG CAA TGG CTA GTG ATC TCT TGA ATC ACT AGC CAT TGC TTT CCG G-3′; shIN-A4293T-S, 5′-GAT CCC GGA GAG CAT TGG CTA GTG ATT CAA GAG ATC ACT AGC CAA TGC TCT CCT TTT TGG AAA G-3′; and shIN-A4293T-AS, 5′-TCG ACT TTC CAA AAA GGA GAG CAT TGG CTA GTG ATC TCT TGA ATC ACT AGC CAA TGC TCT CCG G-3′. To generate pCS-H1-shTat, pCS-H1-shIN, pCS-H1-shING4288A, pCS-H1-shIN-A4293T, and pCS-H1-shU3, pGEM-H1-shTat, pGEM-H1-shIN, pGEM-H1-shING4288A, pGEM-H1-shIN-A4293T, and pGEM-H1-shU3 were digested with EcoRI and SalI. Each fragment was then inserted into the 7.9-kb EcoRI-XhoI fragment of pCS-CDF-PRE.

To introduce the point mutation, T5901C, into the *tat* target sequence of HIV-1 (infectious molecular clone NL-EGFP), total DNA was isolated from MT-4/shTat cells infected by the shTat-resistant HIV-1 variant. The *tat* region of the mutant was amplified by PCR using primers Tat-F (5′-GCA GGA GTG GAA GCC ATA ATA AG-3′) and Tat-R (5′-CAT TAT CAT TCT CCC GCT ACT AC-3′), followed by TA cloning of the PCR product into pT7Blue vector (Merck-Novagen). A 0.28-kb EcoRI-HindIII fragment from the pT7Blue was inserted into pcDNA-NL-RN (pcDNA-TatT5901), which contained a 1.5-kb EcoRI-NheI fragment from NL-EGFP cloned into the EcoRI-NheI sites of pcDNA3.1 (+) (Invitrogen). Finally, the 1.5-kb EcoRI-NheI fragment from pcDNA-TatT5901 was cloned into the EcoRI-NheI site of pNL-EGFP. pNL-EGFP vectors encoding point mutations within the shIN target sequence (G4288A and A4293T) were generated by using a QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol, with mutagenic primers and pNL-EGFP as a template (20). Mutagenic primers were as follows: G4288A, 5′-TCA CAG TAA TTG GAA AGC AAT GGC TAG TG-3′ and 5′-CAC TAG CCA TTG CTT TCC AAT TAC TGT GA-3′; and A4293T, 5′-TCA CAG TAA TTG GAG AGC ATT GGC TAG TG-3′ and 5′-CAC TAG CCA ATG CTC TCC AAT TAC TGT GA-3′. To generate pCS-hU6-shIN50#1, pCS-hU6-shIN50#2, piGENE-hU6-shIN50#1, and piGENE-hU6-shIN50#2 were constructed by inserting the annealing product of shIN50#1 (5′-CAC CGA TGG AGT AGG TAA GGT CCA AGG AGA GCA TGA GGA ATG TCA TAG TAG TTG TTC AAG AGA CAA TTA CTG TGA TAT TTC TCA TGT TCT TCT TGG GCC TTA TCT ATT CCA TCT TTT TT-3′ and 5′-GCA TAA AAA AGA TGG AAT AGA TAA GGC CCA AGA AGA ACA TGA GAA ATA TCA CAG TAA TTG TCT CTT GAA CAA CTA CTA TGA CAT TCC TCA TGC TCT CCT TGG ACC TTA CCT ACT CCA TC-3′) or shIN50#2 (5′-CAC CCA AGA GGA ACG TGA GAG ATA TTA CAG TAG TTG GAG AGT AGT GGC TGG TGA TTC AAG AGA TCA CTA GCC ATT GCT CTC CAA TTA CTG TGA TAT TTC TCA TGT TCT TCT TGT TTT TT-3′ and 5′-GCA TAA AAA ACA AGA AGA ACA TGA GAA ATA TCA CAG TAA TTG GAG AGC AAT GGC TAG TGA TCT CTT GAA TCA CCA GCC ACT ACT CTC CAA CTA CTG TAA TAT CTC TCA CGT TCC TCT TG-3′) into the BspMI site of the piGENE hU6 vector. The EcoRI-PvuII fragment from piGENE-hU6-shIN50-1 or piGENE-hU6-shIN50-2 was inserted into the EcoRI-EcoRV site of pcDNA3.1 (−). The resultant plasmids were digested with EcoRI and XhoI, and the 0.6-kb fragment was ligated into the EcoRI-XhoI site of pCS-CDF-CG-PRE.

Cells. 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μU/ml penicillin, and 100 μg/ml streptomycin. MT-4 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 μU/ml penicillin, and 100 μg/ml streptomycin. Human peripheral blood lymphocytes were derived from HIV-1-seronegative, healthy donors. Briefly, peripheral blood mononuclear cells were separated over a Ficoll-Hypaque gradient (Ficoll-Paque Plus; Amersham Pharmacia Biotech Inc., Tokyo, Japan) by centrifugation. Peripheral blood mononuclear cells were allowed to adhere to 150-mm plastic tissue culture dishes (Iwaki, Tokyo, Japan) by incubation in RPMI 1640 (Sigma Chemical Co., St. Louis, Mo.) containing 5% human AB serum (Sigma or Nippon Bio-SupplyCenter, Tokyo, Japan) for 2 h. Nonadherent cells (peripheral blood lymphocytes) were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2 units of recombinant interleukin-2 (Shionogi, Osaka, Japan)/ml.

Virus preparation. 293T cells (4×10^6) plated in 100-mm dishes were cotransfected with the appropriate lentiviral-shRNA expression vector (17 μg), vesicular stomatitis virus G expression vector pMD.G (5 μg), *rev* expression vector pRSV-Rev (5 μg), and *gag-pol* expression vector pMDLg/pRRE (12 μg) using the calcium phosphate precipitation method. After 4 h, cells were washed three times with phosphate-buffered saline, 5 ml of new medium was added, and cells were incubated for 48 h. Culture supernatants were harvested and filtered through 0.45-μm-pore-size filters. Lentivirus was concentrated ~40-fold by low centrifugation at 6,000 × *g* for 16 h and resuspended in 2 ml of RPMI 1640 medium. In all experiments, cells were transduced with equal amounts of the shRNA lentivirus at a multiplicity of infection of 10. Replication-competent HIV-1 carrying green fluorescent protein (GFP) was generated by transfection of 293T cells with pNL-EGFP (1 μg) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Level of HIV-1 p24 antigen was determined using an enzyme immunoassay (RETRO-TEK; ZeptoMetrix Corp., Buffalo, N.Y.).

Analysis of provirus sequence. Viral DNA was isolated from NL-EGFP-infected MT-4 cells. Viral DNA spanning the shRNA target sequence of interest was amplified by PCR using the following primer pairs: shIN target region primers, 5′-CAC CAT GGG ATT TTT AGA TGG AAT AGA TAA GGC CC-3′ and 5′-ATC CTC ATC CTG TCT ACT TGC-3′; shTat target region primers, 5′-GCA GGA GTG GAA GCC ATA ATA AG-3′ and 5′-CAT TAT CAT TCT CCC GCT ACT AC-3′; and shU3 target region primers, 5′-CGG AAT TCT ACC TTA TC TGG CT-3′ and 5′-TCG CCA CAT ACC TAG AAG AAT AAG AC-3′. These PCR products were inserted into the pGEM-T Easy vector (Promega) by TA cloning, followed by DNA sequence analysis using the ABI310 sequencer (Perkin-Elmer Applied Biosystems).

Quantitative PCR analysis. Total DNA was extracted from cells 1 or 8 days postinfection by using the urea lysis method. Briefly, cells were lysed with 0.3 ml of urea lysis buffer (7 M urea, 2% sodium dodecyl sulfate, 1 mM EDTA, 10 mM Tris-HCl [pH 8.0], 0.35 M NaCl). Total DNA was purified from the cell lysates by phenol-chloroform extraction followed by ethanol precipitation. Analysis of HIV-1 DNA was performed by quantitative PCR with the HIV-1-specific primers vif-F (5′-GAG ATA TAG CAC ACA AGT AGA CC-3′) and vif-R (5′-GCT AGT GCC AAG TAC TGT GAG AT-3′) using *Taq* DNA polymerase (Invitrogen). The thermal cycle consisted of 1 min at 94°C, followed by 30 cycles of 94°C for 1 min (denaturation), 65°C for 2 min (annealing), and 72°C for 2 min (extension). PCR products were separated on 2% agarose gels and stained with SYBR green.

RESULTS

Inhibition of HIV-1 replication by lentiviral-shRNA targeting of *tat*, integrase, and U3 *att* sequences. Upon HIV-1 infection, the viral enzyme integrase catalyzes the integration of viral DNA into the host cell chromosome, an obligatory step for HIV-1 gene expression. In preliminary experiments using synthetic siRNA duplexes targeting essential motifs within HIV-1 IN (17, 27, 35), we identified several candidate sequences for shRNA-mediated targeting of HIV-1 (data not shown). Earlier studies showed that introduction of a single-amino-acid substitution within the HHCC motif of HIV-1 IN completely abolished virus infectivity (27, 29), indicating that sequence variation in this region is not tolerated by the virus. Indeed, the selected sequences are highly conserved among

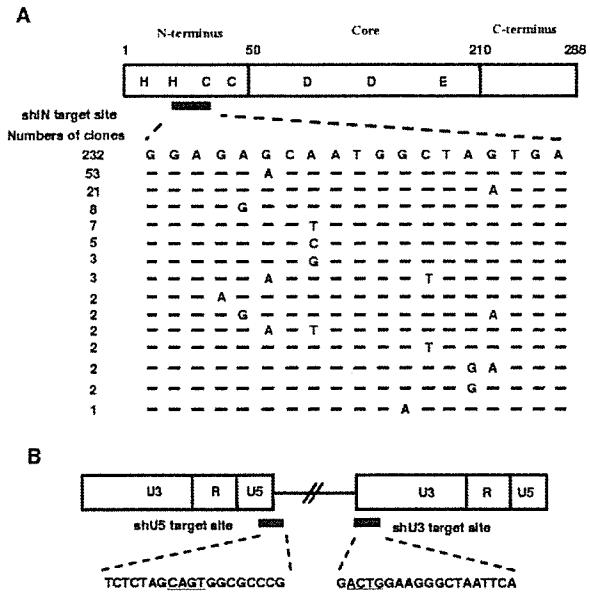

FIG. 1. shRNA target sequences of HIV-1 *int* and the U3 and U5 *att* sites. (A) Schematic representation of HIV-1 integrase (top squares) and shIN target region (bold bar). The conserved HHCC residues in the zinc-binding motif and enzyme active-site residues (DDE) located in the N terminus of the core domain of HIV-1 IN are indicated. The sequence of the shIN target region corresponding to the HIV-1 NL43 clone (1) and used in the present study is shown on the first line. Sequences of this region in 345 different isolates were aligned according to the HIV-1 sequence database published by Los Alamos National Laboratory (http://hiv-web.lanl.gov). Nucleotide differences in comparison with NL43 and numbers of clones that carried them are indicated. (B) Target sequences of shU3 and shU5 in the HIV-1 NL43 clone are shown. Location of each target region in a whole HIV-1 genome is indicated by the bold bar. Conserved sequences in the U3 *att* and U5 *att* regions among all HIV-1 strains are underlined.

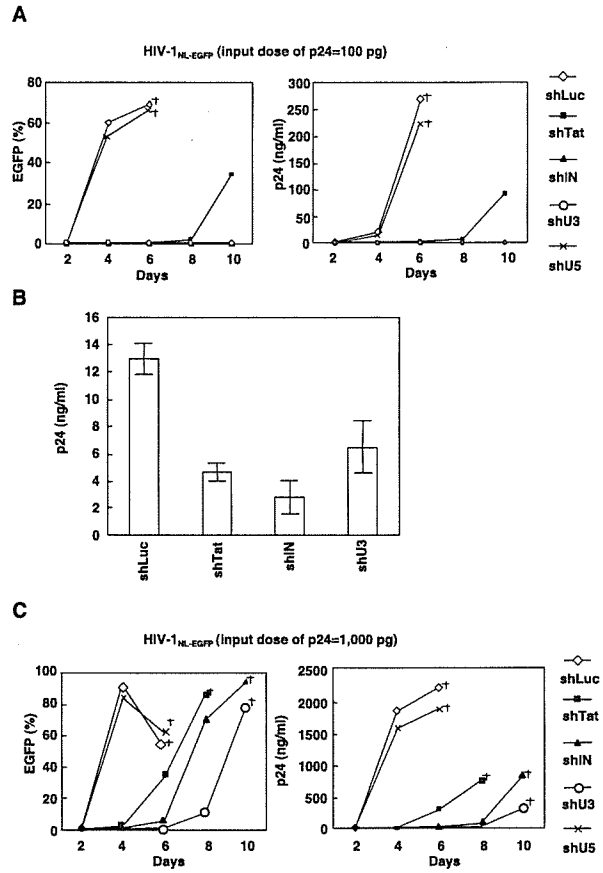

FIG. 2. HIV-1 replication in shRNA-transduced MT-4 or primary $CD4^+$ T cells. (A) MT-4 cells were transduced by the indicated shRNA using a lentivirus vector system (shLuc, shTat, shIN, shU3, and shU5). Seven days later, transduced MT-4 cells were infected by $HIV\text{-}1_{NL\text{-}EGFP}$ at an input dose of 100 pg of p24 (HIV-1 core antigen) per 10^6 cells. Culture supernatants were collected periodically after infection as indicated. HIV-1 replication was monitored by measuring percent EGFP-positive cells by fluorescence-activated cell sorting (left) or level of HIV-1 p24 by enzyme-linked immunosorbent assay (right). The cross symbol indicates cell death associated with HIV-1 replication. Representative results of three independent experiments are shown. (B) Inhibition of HIV-1 replication by each shRNA in primary $CD4^+$ T cells. $CD4^+$ T cells (1×10^6) were transduced with the indicated lentiviral vectors by low centrifugation at 2,000 rpm for 1 h in the presence of 10 μg/ml polybrene. Transduced cells were infected with $HIV\text{-}1_{NL43}$ for 3 h, and p24 antigen levels in culture supernatants 4 days postinfection were measured. Values represent the means and standard deviations for three independent experiments. (C) MT-4 cells transduced with the indicated shRNAs (shLuc, shTat, shIN, shU3, or shU5) were infected by $HIV\text{-}1_{NL\text{-}EGFP}$ at a high input dose (1,000 pg of p24 per 10^6 cells), and HIV-1 replication was monitored as described for panel A. (C) MT-4 cells transduced with each shRNA (shLuc, shTat, shIN, shU3, or shU5) were infected by $HIV\text{-}1_{NL\text{-}EGFP}$ with a high input dose (1,000 pg of p24 per 10^6 cells), and HIV-1 replication was monitored as described for panel A.

HIV-1 strains (Fig. 1A). Therefore, we chose this site for lentiviral-shRNA-mediated gene targeting. We also chose the conserved regions within the U3 *att* and U5 *att* sites as the shRNA targets (Fig. 1B). The attachment sites at both viral DNA ends (U3 *att* and U5 *att*) are *cis*-acting regions required for retroviral integration by IN, and point mutations or deletion of HIV-1 U3 or U5 *att* sites resulted in severe impairment of integration in vivo (25, 27). The lentiviral-based shRNA expression system we used in these studies was previously shown to efficiently inhibit HIV-1 replication in 293T and MT-4 cells and primary macrophages (30).

First, we examined the effect of expressing shRNAs targeting IN (shIN), U3 *att* (shU3), or U5 *att* (shU5) on HIV-1 replication in a highly susceptible human $CD4^+$ T-cell line, MT-4 (15). The MT-4 cell system has been used successfully by us and others to isolate HIV-1 mutants acquired with resistance to the neutralization antibody (26) or anti-HIV-1 drugs (13). As a positive control for shRNA-mediated inhibition of HIV-1, we constructed a lentivirus vector expressing shRNAs for the HIV-1 transactivator protein gene *tat* (shTat) that corresponded to the region described by Boden et al. (6). Following transduction with lentiviral shRNAs, MT-4 cells were infected at various doses with an HIV-1 clone carrying enhanced GFP (pNL-EGFP) (20). Expression of shIN, shU3, and shTat profoundly inhibited HIV-1 replication compared with the control shRNA, shLuc, at an input dose of 100 pg of HIV-1 p24 (*gag* gene product), or the equivalent of a 50% tissue culture infective dose of about 200 (Fig. 2). The inhibitory effect of shU5 expression was very weak, possibly due to a high G+C content in its 3′ region (36). At 10 days postinfection, viral replication was detected in the shTat-transformed MT-4 cells,

followed by cell death. In contrast, HIV-1 replication was undetectable in MT-4 cells transduced by shIN or shU3 up to 1 month postinfection, indicating complete inhibition of HIV-1 by shIN or shU3 during this time frame. Thus, in MT-4 cells, shIN and shU3 conferred stronger resistance against HIV-1 than shTat. The antiviral effect of each shRNA was also observed in human primary $CD4^+$ T cells (Fig. 2B), where shIN exhibited the strongest antiviral activity. Prolonged antiviral activity by shIN or shU3 was abolished by increasing the level of input HIV-1 to 1,000 (Fig. 2C) or 10,000 pg of p24 (not shown). Under conditions of increased infectious dose, HIV-1 replication was observed 10 days postinfection in MT-4 cells transduced with shIN or shU3.

Genetic analysis of shRNA-resistant HIV-1. Although each shRNA could inhibit HIV-1 replication under conditions of low dose of infection, the inhibitory effect was transient when higher input doses were used. This effect was most likely due to acquired mutations within the viral shRNA target sequences. Viruses were harvested from MT-4 cells that had been transduced by each of the shRNAs and used to infect a fresh set of shRNA-transduced MT-4 cells. Viruses harvested from culture supernatants 12 days after infection of shTat-transduced MT-4 cells showed specific resistance against shTat but not against shIN or shU3 (Fig. 3A, left). Viruses harvested from shIN- or shU3-transduced MT-4 cells 10 days after infection with high doses of HIV-1 also showed specific resistance against shIN or shU3 *att*, respectively (Fig. 3B and C, left).

We next examined the genetic profile of shRNA target sites in each shRNA-resistant virus. MT-4 cells were freshly infected with each shRNA-resistant virus, and total DNA was extracted. Viral DNA fragments spanning each shRNA target region were amplified by PCR, followed by TA cloning. Several clones derived from each of the resistant viruses were examined by DNA sequence analysis. Various single-nucleotide substitutions were observed within the shTat target region of shTat-resistant virus DNA (Fig. 3A, right), while the sequences within the shIN and shU3 target regions were unchanged (not shown). Similarly, shIN- or shU3-resistant viruses contained one or two mutations within the corresponding target region. No viruses in which wild-type sequences in each shRNA target region were maintained emerged after long-term culture (16 to 22 days postinfection), indicating a strong selective pressure of these shRNAs toward wild-type virus. Of note, shIN-resistant viruses contained only two types of mutation (G4288A and A4293T), suggesting that mutations in the IN region are more detrimental for virus replication than those in other shRNA targeted regions in *tat* and U3 *att*.

To confirm whether the nucleotide substitutions detected in the above experiments could confer resistance to the corresponding shRNA, we introduced each point mutation into the parental HIV-1 clone (pNL-EGFP) and evaluated its replication ability in shRNA-transduced MT-4 cells. Viruses carrying point mutations within the shTat target site (Tat-T5901C) or the shIN target site (IN-G4288A or IN-A4293T) showed specific resistance against shTat or shIN, respectively (Fig. 4). We also observed that IN-G4288A or IN-A4293T mutants had constantly higher levels of replication in MT-4 cells transduced with shIN than in control MT-4 cells transduced by shLuc (Fig. 3B and C). Although the mechanism underlying the enhanced replication of these escape mutants in the presence of shIN is unknown, enhancement of HIV-1 replication by siRNA has been reported recently by others (10). These results indicate that shRNA-mediated selection pressure can generate HIV-1 escape mutants that can replicate in the presence of each shRNA.

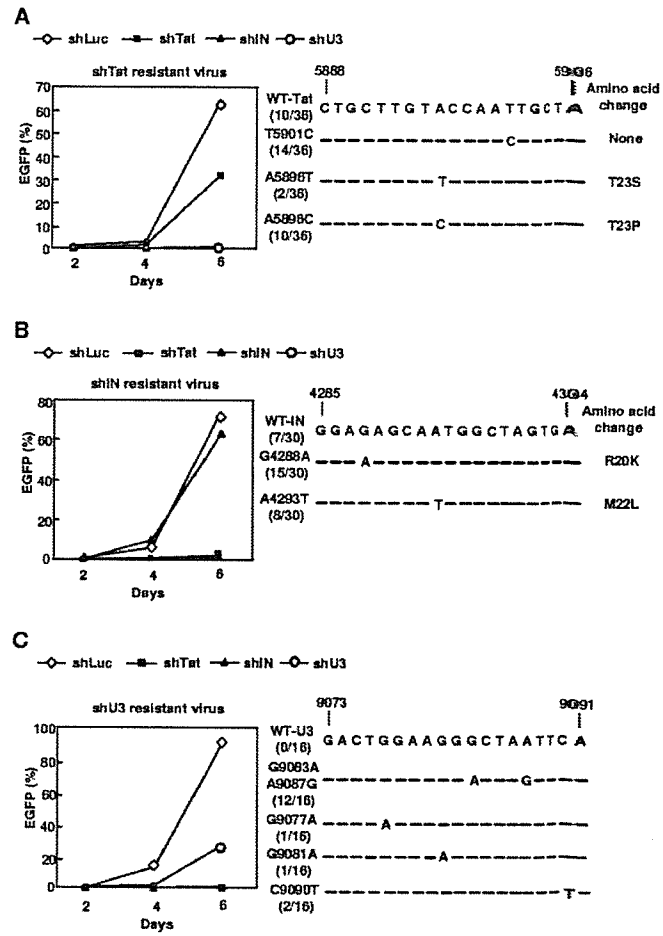

FIG. 3. shRNA-specific resistance of HIV-1 escape mutants in shRNA-transduced MT-4 cells. Culture supernatants of shTat-transduced MT-4 cells infected with a low dose of HIV-1 (100 pg of p24) (A) or shIN (B)- or shU3 (C)-transduced MT-4 cells infected with a high dose of HIV-1 (1,000 pg of p24 per 10^6 cells) were harvested at 12 days after challenge infection. Culture supernatants containing shRNA-resistant virus (100 pg of p24) were inoculated to newly prepared MT-4 cells transduced by shLuc, shTat, shIN, or shU3, and replication of HIV-1 in these cells was monitored by measuring percent EGFP-positive cells (left). Representative results of three independent experiments are shown. Culture supernatants of shTat-transduced MT-4 cells infected with shTat-resistant virus (A), shIN-transduced MT-4 cells infected with shIN-resistant virus (B), and shU3-transduced MT-4 cells infected with shU3-resistant virus (C) were harvested at 6 days postinfection. Each culture supernatant containing shRNA-resistant viruses (100 pg of p24) was infected with newly prepared MT-4 cells transduced by shTat, shIN, or shU3. Total DNA was extracted from these MT-4 cells at 4 days postinfection. A fragment of viral DNA spanning each shRNA target region was amplified by PCR followed by TA cloning. Then, several clones from each were subjected to DNA sequence analysis. Nucleotide changes in the target sequence for shTat (nucleotides 5888 to 5906 of the *tat* gene), shIN (nucleotides 4285 to 4304 of the *int* gene), and shU3 (nucleotides 9073 to 9091 of the U3 *att* region) are shown on the right, along with the expected amino acid changes. Relative numbers of each clone are indicated in parentheses.

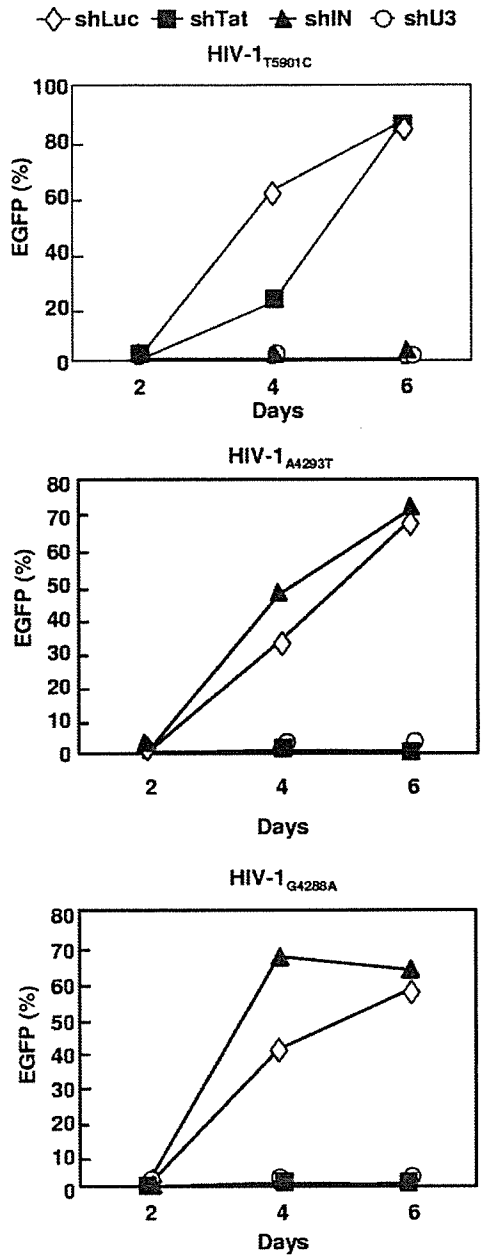

FIG. 4. shRNA-specific resistance of HIV-1 molecular clones carrying point mutations within each shRNA target site. Point mutations within the shTat target site (Tat-T5901C) or shIN target site (IN-G4288A or IN-A4293T) were introduced into the parental HIV-1 clone (pNL-EGFP) through mutagenesis. Each recombinant mutant clone was transfected into 293T cells, and the culture supernatant was harvested and inoculated to MT-4 cells expressing the corresponding shRNA. Replication of each mutant clone was monitored by measuring percent EGFP-positive cells at the indicated days. Representative results of three independent experiments are shown.

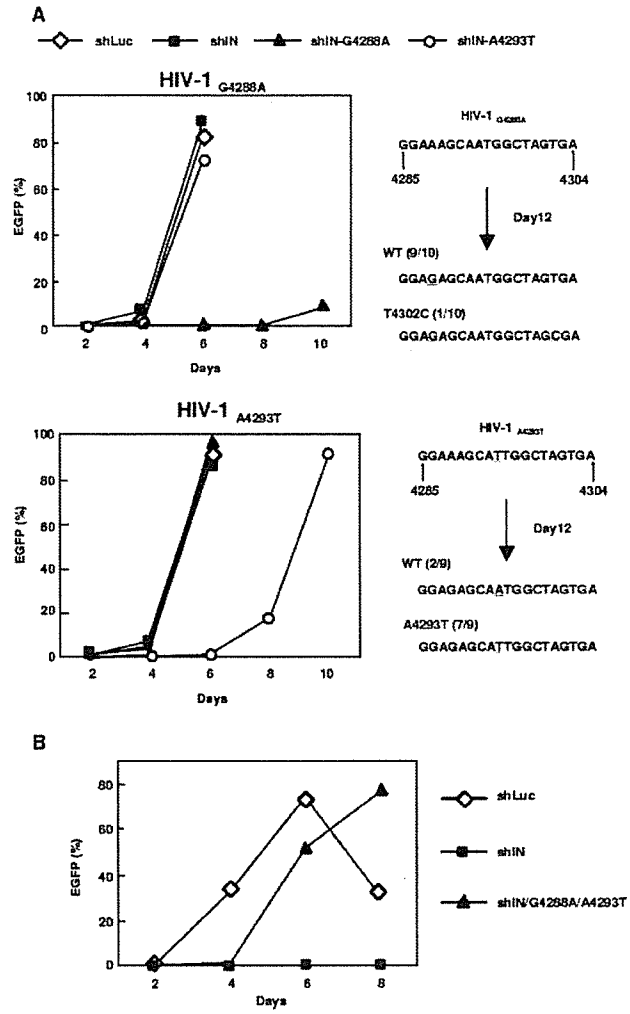

FIG. 5. Reversion of escape mutants in the presence of modified shRNAs targeting mutant sequences. (A) MT-4 cells were transduced with shIN or its modified shIN, which targeted escape mutant sequences (shIN-G4288A or shIN-A4293T). As a negative control, shLuc was introduced into MT-4 cells as well. The transduced cells were infected by HIV-$1_{NL\text{-}G4288A}$ or HIV-$1_{NL\text{-}A4293T}$ at a dose of 1,000 pg of p24 antigen per 10^6 cells. Ten or 12 days after challenge infection, total DNA was isolated, viral DNA spanning the shIN target region was amplified by PCR and subjected to TA cloning, and sequences were analyzed. WT, wild type. (B) Effect of sequential transduction of shRNAs targeting *int* from wild-type and escape mutant viruses. MT-4 cells were transduced with shIN, shIN-G4288A, and shIN-T4293A sequentially (shIN/G4288A/T4293A). In parallel, MT-4 cells were transduced with shIN or shLuc alone. Transduced MT-4 cells were infected with HIV-$1_{NL\text{-}EGFP}$ at a dose of 100 pg of p24 per 10^6 cells. Virus replication was monitored by measuring percent EGFP-positive cells at the indicated days. Representative results of three independent experiments are shown.

Combination of shINs against wild-type and escape mutants. Two different single-nucleotide substitutions were identified in shIN escape mutants (G4288A and A4293T). We examined HIV-1 replication in MT-4 cells expressing shRNAs targeting wild-type IN and both of the variant sequences (G4288A and A4293T). We constructed shRNA expression vectors encoding the IN escape mutant sequences, shIN-G4288A and shIN-A4293T, and confirmed their specific abilities to suppress the replication of the corresponding viral mutants, IN-G4288A and IN-A4293T, respectively (Fig. 5A). However, significant viral replication was detected 8 to 10 days postinfection, with IN-G4288A or IN-A4293T in MT-4 cells expressing the corresponding mutant shRNA (Fig. 5A). Sequence analysis of clones isolated from shIN-G4288A-trans-

duced MT-4 cells had revealed that 9 out of 10 had wild-type IN sequences, suggesting that these viruses had reverted to the wild type. In nine clones isolated from shIN-A4293T-transduced MT-4 cells, two had IN sequences that had reverted to the wild type, and seven clones retained the original mutation (Fig. 5A, lower panel). Differences in the efficiencies of reversion of the two mutants may reflect different selection pressures conferred by shIN-G4288A or shIN-A4293T.

Reversion to wild-type sequences was detected only when each escape mutant was treated with its corresponding mutant-specific shRNA. We next examined the effect of combining shRNAs targeting the wild-type and shIN escape mutant viruses on the emergence of mutant and/or wild-type virus. MT-4 cells were sequentially transduced with shIN, shIN-G4288A, and shIN-A4293T and then infected with wild-type HIV-1. Contradictory to our expectations, the combination of two different shRNAs weakened HIV-1 suppression by shRNAs (Fig. 5B). We detected significant HIV-1 replication 6 days postinfection, under the same conditions that resulted in complete suppression by shIN alone. Sequence analysis revealed that only wild-type HIV-1 had persisted, and escape mutant viruses were not detected (data not shown). These experiments suggest that several shRNAs targeting the same region might have a detrimental effect on their suppression capabilities, perhaps due to competition between the same target RNAs, with less effective shRNAs carrying a mismatch point mutation.

The combination of shRNA targeting different sites of HIV-1 for efficient suppression of HIV-1. We next evaluated the antiviral effect of combining shRNAs that target different sites within the HIV-1 genome. MT-4 cells were simultaneously transduced with three different combinations of shRNAs: shIN/shU3, shTat/shU3, and shTat/shIN. The transduced MT-4 cells were infected with a dose of HIV-1 containing 1,000 pg of p24 antigen. These were the conditions under which a single type of shRNA could not control viral replication and escape mutants emerged (Fig. 2). All of the combinations of shRNAs completely inhibited HIV-1 replication, and the inhibitory effect persisted for more than 1 month without emergence of escape mutants (Fig. 6A). These results demonstrated that shRNAs targeting at least two different essential genes might have a positive impact on suppressing viral activity.

Interestingly, we observed that proviral DNA was absent in dual-transformed MT-4 cells after HIV-1 infection. We speculated that the shRNAs might target and degrade incoming viral RNA, preventing subsequent viral cDNA synthesis. We analyzed the levels of viral cDNA synthesized soon after HIV-1 infection of shRNA-transduced MT-4 cells by a quantitative PCR, using primers specific for the type of HIV-1 used for the challenge infection. In control MT-4 cells transduced with shLuc, viral cDNA was detected as early as 6 h postinfection. The levels of viral cDNA increased over time, indicating multiple rounds of viral infection (Fig. 6B). In contrast, at 6 h postinfection, the levels of viral cDNA in MT-4 cells transduced with shIN/shU3, shTat/shU3, or shTat/shIN were significantly reduced to 45%, 49%, or 15%, respectively, of those in control MT-4 cells and then declined to undetectable levels at 24 h and the later time point (8 days) after infection. These results suggested that shRNAs could target incoming viral RNA, thereby preventing subsequent reverse transcription and integration of HIV-1 RNA.

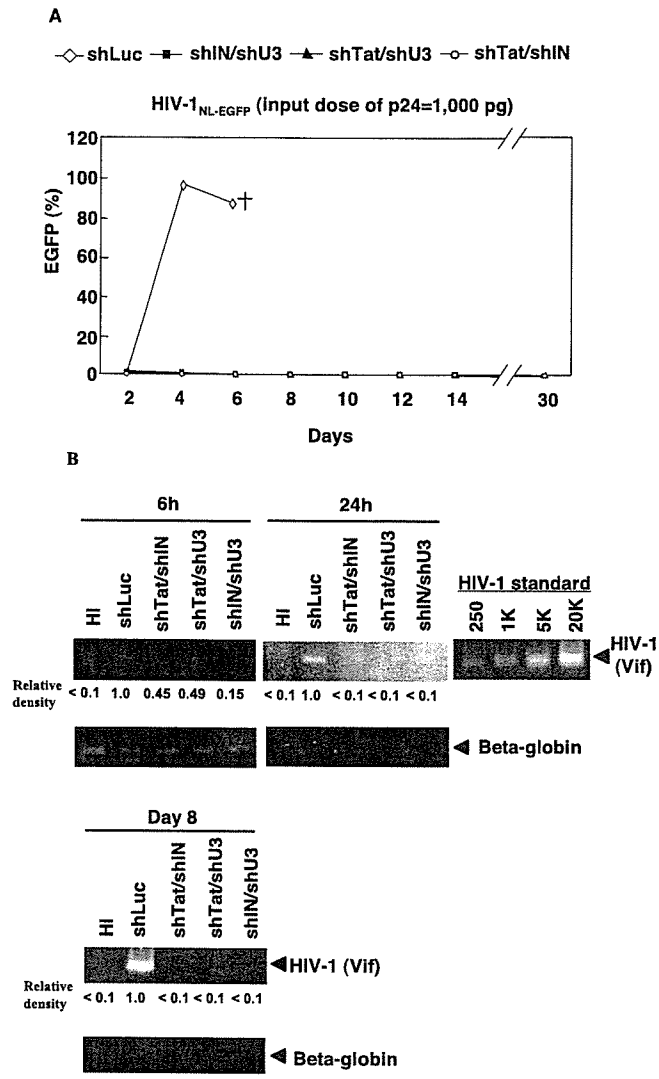

FIG. 6. Efficient antiviral activity with a combination of shRNAs targeting different sites in the HIV-1 genome. (A) MT-4 cells were transduced with combinations of shIN and shU3 (shIN/shU3), shTat and shU3 (shTat/shU3), or shTat and shIN (shTat/shIN). The dual-transduced cells were infected with DNase I-treated HIV-$1_{NL-EGFP}$ at a dose of 1,000 pg of p24 per 10^6 cells. Virus replication was monitored by measuring percent EGFP-positive cells at the indicated days postinfection. The cross symbol indicates cell death associated with HIV-1 replication. (B) In parallel, total DNA was isolated from MT-4 cells 6 h, 24 h, or 8 days postinfection. Level of viral DNA was determined by quantitative PCR as described previously (27). For PCR, virus incubated at 65°C for 30 min prior to inoculation was used as the heat-inactivated control (HI), and for the HIV-1 DNA standard, a linearized HIV-1 molecular clone (pNL43lucΔ*env*) was amplified. Human β-globin DNA was used as the internal control (17). The gel image was taken by using Image Saver System AE-6905C (ATTO, Tokyo, Japan), and the intensities of the PCR products were quantified by using Adobe Photoshop 7.0 software. The values shown are the intensity of each band relative to that in the control shLuc-transduced MT-4 cells, taken as 1.0.

For successful long-term control of HIV-1 replication by shRNA, targeting the incoming viral RNA before reverse tran-

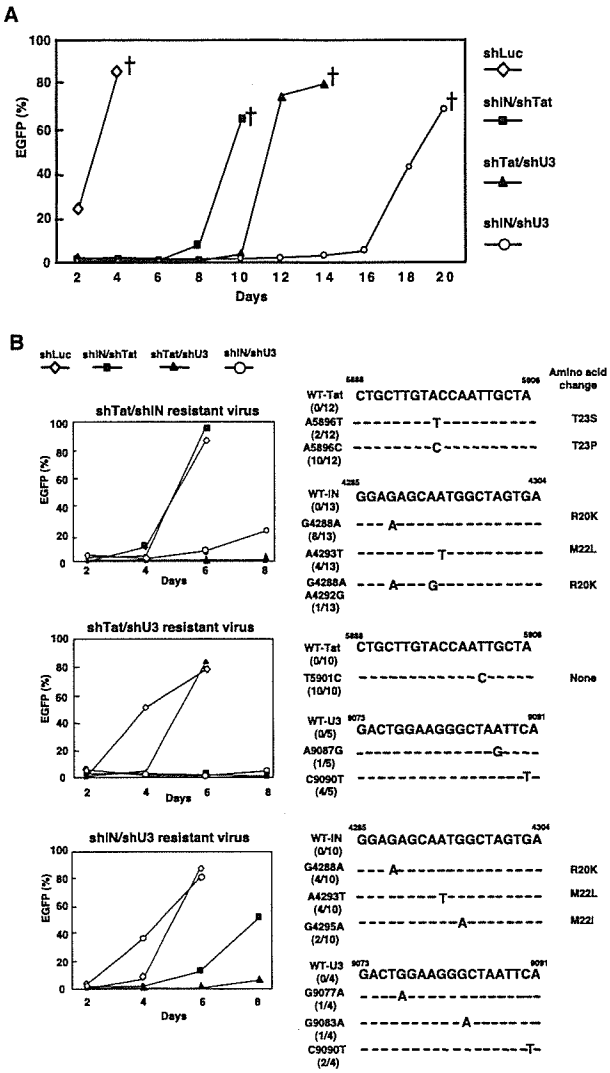

FIG. 7. Emergence of escape mutants from combinations of shRNAs targeting different sites following a high dose of HIV-1 infection. (A) MT-4 cells were transduced with combinations of two shRNAs as described for Fig. 6. The dual-transduced cells were infected with HIV-$1_{NL\text{-}EGFP}$ at 10,000 pg of p24 per 10^6 cells. Virus replication was monitored by measuring percent EGFP-positive cells at the indicated days postinfection. (B) Viruses were harvested from the culture supernatants of dual-transduced MT-4 cells 12 days postinfection for shTat/shIN, 16 days for shTat/shU3, and 22 days for shIN/shU3. Viruses resistant to each combination of shRNAs were inoculated into MT-4 cells freshly transduced by each combination of the two shRNAs. Virus replication was monitored by measuring percent EGFP-positive cells at the indicated days. The cross symbol indicates cell death associated with HIV-1 replication. Total DNA was harvested from the infected cells when virus replication became evident. Viral DNA spanning each shRNA target region was amplified by PCR and subjected to TA-cloning followed by sequence analysis, as described for Fig. 3. WT, wild type.

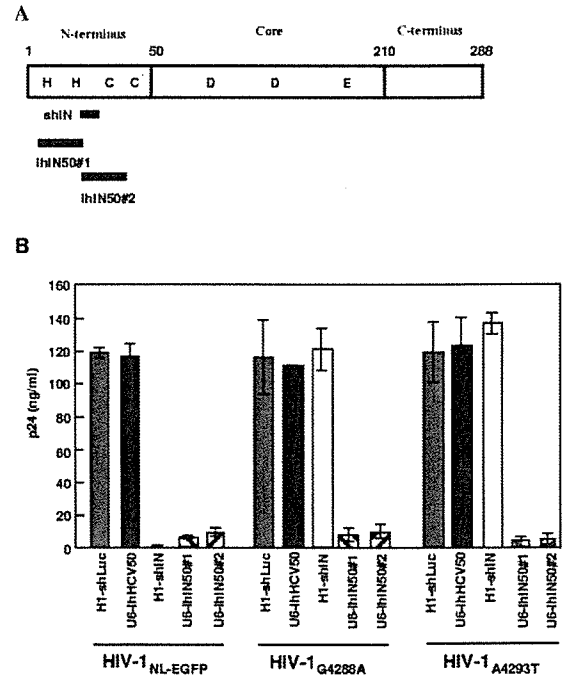

FIG. 8. Inhibitory effect of lhRNA on wild-type or shIN-resistant viral clones. (A) The target sites of lhRNAs against the HIV-1 *int* gene (lhIN). The target sites of two lhINs (lhIN50#1 and lhIN50#2) are indicated by bold bars. lhIN#1 was designed to target the 50 nucleotides upstream of the shIN target sequence. lhIN50#2 targets 50 nucleotides that include the shIN target sequence. (B) Lentiviral vectors expressing each lhRNA under the control of the human H1 promoter or U6 promoter were constructed. As a negative control, lhRNA targeting 50 nucleotides of HCV genome (U6-lhHCV50) was used (38). Transduction of MT-4 cells with each lhRNA was performed as described for Fig. 2. Transduced MT-4 cells were infected by the parental clone (HIV-$1_{NL\text{-}EGFP}$) or the shIN-resistant clone (HIV-1_{G4288A} or HIV-1_{A4293T}) at a dose of 100 pg of p24 per 10^6 cells. Virus replication was monitored by measuring levels of p24 antigen in culture supernatants 4 days postinfection. Values are the means plus standard deviations for three independent experiments.

scription might be a key point of interference. In support of this hypothesis, when the infectious dose of HIV-1 was increased to the level of 10,000 pg of p24 antigen, none of the combinations of shRNAs was able to control HIV-1 replication in long-term cultures (Fig. 7A). When we examined the viruses replicating in the presence of each combination of shRNA, they showed specific resistance against the corresponding shRNAs and corresponding genetic alterations within both shRNA target sites (Fig. 7B). Note, however, that the combination of shIN/shU3 showed the strongest suppressive effects, inhibiting viral replication until 18 days postinfection, with a high dose of HIV-1 in the challenge infection (Fig. 7A).

Modified long-hairpin RNA can suppress replication of HIV-1 wild-type or shRNA escape mutants in short-term culture. Recently, it was reported that modified lhRNA, in which multiple point mutations were introduced into the sense strand to prevent activation of the cellular-interferon response (28), could effectively suppress the replication of hepatitis C virus (38). We constructed lentiviral vectors expressing lhRNAs targeting 50 nucleotides that span the shIN target region of the *int* gene (Fig. 8A). When lentiviral lhRNAs were expressed under the control of the human H1 promoter, viral replication was not significantly inhibited, perhaps due to the low expression levels and/or low stability of the transcripts (data not shown). Therefore, we used the human U6 promoter to drive lhRNA expression and evaluated the antiviral activity of the lhRNAs against wild-type or shIN-resistant clones (Fig. 8B). As described above, shIN had antiviral activity against wild-type

HIV-1 but not against the shIN-resistant clones (IN-G4288A or IN-A4293T). In contrast, lhRNAs targeting the *int* gene efficiently blocked replication of both of wild-type virus and the shIN-escape mutants (Fig. 8B). Interestingly, the anti-HIV-1 activity of lhIN50#2 was similar to that of lhIN50#1, which did not contain shIN target sequences, suggesting that viruses could not escape from RNAi caused by the lhRNAs. However, the antiviral effects of lhIN50#1 and lhIN50#2 were transient, and low levels of viral replication were detected 6 days postinfection. Sequence analysis revealed that replicating viruses were genotypically wild type (data not shown). Thus, the antiviral activity of the lhRNAs was not strong enough to induce generation of escape mutants, perhaps due to the low expression levels or poor stability of the expressed lhRNAs. The development of a more efficient expression system for lhRNAs might be necessary to achieve long-term control of HIV-1 replication. Nonetheless, our data suggest that targeting longer sequences of HIV-1 could be beneficial and an alternative approach to suppressing escape mutants.

DISCUSSION

Expression of siRNAs directed against viral RNA has a potent and sequence-specific antiviral effect. However, viruses can escape from RNAi because of their high mutation rate. One approach to designing an effective siRNA-based therapy against HIV-1 is to target highly conserved regions in the HIV-1 genome. In this study, we showed that HIV-1 replication was efficiently inhibited through the expression of shRNAs that targeted the *int* or U3 *att* region, with no emergence of shRNA escape mutants when low doses of infection were used. However, shRNA escape mutant viruses did emerge with a higher dose of HIV-1 infection. Notably, among the target sequences examined in these studies, the target site for shIN is potentially the least-mutated region of the HIV genome.

Recently, it was shown that accumulation of several point mutations is required for siRNA resistance in an HCV replicon system (40). Several studies have suggested that shRNA-resistant virus can emerge not only by escaping the siRNA-mediated degradation of mRNA but also by micro RNA-mediated translational inhibitory pathways (8, 19, 24, 37, 41). In this paper, we showed that a single point mutation within a target site is sufficient for HIV-1 to escape from shRNA-mediated inhibition. This difference between HIV-1 and HCV might be partly due to suppressor protein function in RNA silencing. HCV has not been shown to encode a suppressor protein for RNA-silencing function, such as HIV-1 Tat (5) or influenza virus NS1 (9). One of the escape mutants in these studies showed enhanced replication in the presence of shRNA (Fig. 3, shIN-resistant virus clone). Similar enhancement by shRNA was also noted by others (10), sounding a cautionary note that if not selected properly, siRNA may enhance, rather than inhibit, virus replication.

The experiments in which several combinations of shRNAs were used revealed important new clues towards understanding siRNA-based therapeutic approaches against HIV-1. Pretreatment of cells simultaneously with shINs targeting wild-type and escape mutant sequences to prevent the emergence of escape mutations resulted in HIV-1 replication of wild-type sequences. Thus, there appears to be a detrimental effect of simultaneously administering shRNAs that target overlapping sequences in an effort to cover variant sequences among different HIV-1 strains. In contrast, multiple shRNAs targeting different essential sequences had a strong impact on antiviral activity.

HIV-1 Tat possesses a suppressor of RNA silencing function to evade elicited RNAi. Importantly, Tat suppresses RNAi mediated by shRNAs but not by synthesized oligonucleotide siRNA duplexes. shRNA requires Dicer-mediated processing to elicit RNAi, whereas presynthesized siRNA does not, suggesting that the role of Tat may be to subvert the cell's Dicer activity and inhibit processing of precursor double-stranded RNAs into siRNAs (5). Therefore, we were interested in testing other siRNAs against the HIV-1 genome in combination with siRNA targeting the *tat* gene. A synergic effect of shTat in combination with either shIN or shU3 was not detected in our studies. Rather, a combination of shIN and shU3 was shown to be most effective against HIV-1. Thus, we demonstrated a positive impact on the antiviral effect of shRNAs by using combinations of siRNAs targeting different regions of the genome. The lhRNAs, which targeted longer sequences, were also effective against viral pools containing divergent sequences or escape mutant sequences. Our lhRNA system, however, needs further modification to increase the expression and/or stability of the precursor transcripts. Taken together, the results of the present study suggest that targeting incoming viral RNA before viral cDNA synthesis through multiple or longer siRNAs is an important key for successful RNAi-mediated antiviral therapy.

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Reduction of Human T-Cell Leukemia Virus Type 1 (HTLV-1) Proviral Loads in Rats Orally Infected with HTLV-1 by Reimmunization with HTLV-1-Infected Cells

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Human T-cell leukemia virus type 1 (HTLV-1) persistently infects humans, and the proviral loads that persist in vivo vary widely among individuals. Elevation in the proviral load is associated with serious HTLV-1-mediated diseases, such as adult T-cell leukemia and HTLV-1-associated myelopathy/tropical spastic paraparesis. However, it remains controversial whether HTLV-1-specific T-cell immunity can control HTLV-1 in vivo. We previously reported that orally HTLV-1-infected rats showed insufficient HTLV-1-specific T-cell immunity that coincided with elevated levels of the HTLV-1 proviral load. In the present study, we found that individual HTLV-1 proviral loads established in low-responding hosts could be reduced by the restoration of HTLV-1-specific T-cell responses. Despite the T-cell unresponsiveness for HTLV-1 in orally infected rats, an allogeneic mixed lymphocyte reaction in the splenocytes and a contact hypersensitivity response in the skin of these rats were comparable with those of naive rats. HTLV-1-specific T-cell response in orally HTLV-1-infected rats could be restored by subcutaneous reimmunization with mitomycin C (MMC)-treated syngeneic HTLV-1-transformed cells. The reimmunized rats exhibited lower proviral loads than untreated orally infected rats. We also confirmed that the proviral loads in orally infected rats decreased after reimmunization in the same hosts. Similar T-cell immune conversion could be reproduced in orally HTLV-1-infected rats by subcutaneous inoculation with MMC-treated primary T cells from syngeneic orally HTLV-1-infected rats. The present results indicate that, although HTLV-1-specific T-cell unresponsiveness is an underlying risk factor for the propagation of HTLV-1-infected cells in vivo, the risk may potentially be reduced by reimmunization, for which autologous HTLV-1-infected cells are a candidate immunogen.

Human T-cell leukemia virus type 1 (HTLV-1) is a human retrovirus associated with adult T-cell leukemia (ATL) and a variety of chronic inflammatory diseases including HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (5, 12, 38, 41). Although a small proportion of HTLV-1-infected individuals develop ATL after a long latency (24, 30, 46), most affected individuals remain asymptomatic during their lifetime. ATL is a highly aggressive $CD4^+$ T-cell leukemia/lymphoma characterized by clonal integration of HTLV-1 in leukemic or lymphoma cells (53). Although the precise mechanism of leukemogenesis in ATL remains unclear, several etiological risk factors have been suggested, including vertical transmission, gender (males more than females), and an increase in the number of abnormal lymphocytes associated with a high HTLV-1 proviral load (13, 14, 37, 44).

In an infected person, the proviral load of HTLV-1 is usually stable over time (32). However, what determines the set point of the proviral load in each person is not well understood. Several studies on HAM/TSP patients and HTLV-1-carriers have indicated that there is a weak positive correlation between the frequency of HTLV-1-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) and the proviral load of HTLV-1 (26, 51). Meanwhile, other studies have reported that circulating $CD8^+$ CTLs from individuals with a low HTLV-1 proviral load express greater levels of genes that encode granzymes and other lytic proteins than the corresponding cells in individuals with a high proviral load (48). A theoretical model has been proposed that the efficacy of $CD8^+$ CTLs determines the level of the set point and that the equilibrium frequency of virus-specific $CD8^+$ CTLs is the same between individuals with lower and higher viral loads (35).

The level of $CD8^+$ CTL activity against HTLV-1 varies widely among HTLV-1-infected individuals. High levels of HTLV-1-specific CTL activity are observed in HAM/TSP patients and some asymptomatic HTLV-1 carriers (16, 21, 40). In contrast, ATL patients are apparently defective for HTLV-specific CTL activity, although it can be sporadically induced during the remission stages or only after mitogenic stimulation with multiple in vitro antigenic stimulations of peripheral blood mononuclear cells (1, 20). HTLV-1-specific CTLs mainly recognize Tax (16, 18), a molecule responsible for T-cell immortalization (17, 52), and CTLs induced in ATL patients in remission are able to lyse autologous tumor cells in vitro (19). These observations suggest that HTLV-1-specific CTLs play a crucial role in host immunosurveillance against ATL cells. In support of this notion, Tax-specific CTLs can eradicate HTLV-1-infected tumors in a rat model of ATL-like HTLV-1-associated lymphoproliferative disease (7).

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The reasons for the insufficient HTLV-1-specific CTL responses in ATL patients are not clear. Recent reports have indicated that the phenotype of ATL cells resembles that of regulatory T cells, although their functional properties do not fully match those of regulatory T cells (3). Vertical HTLV-1 transmission, one of the epidemiological risk factors for ATL, may cause insufficiency in the HTLV-1-specific T-cell response. Vertical HTLV-1 transmission mainly occurs through breast-feeding from HTLV-1-carrying mothers (10), since intervention by refraining from breast-feeding was found to block >80% of vertical transmission of HTLV-1 (9). Both oral intake and exposure at a young age may induce immune tolerance against the exposed antigens (47).

We previously reported that the HTLV-1-specific cellular and humoral immunities of orally HTLV-1-infected rats were impaired compared to those of intraperitoneally infected rats (22). In contrast, the HTLV-1 proviral load of orally infected rats was significantly greater than that of intraperitoneally infected rats. These findings indicate that oral HTLV-1 infection induces insufficient host immune conditions that favor viral expansion. Since HTLV-1 is mainly associated with infected cells, an increase in the proviral load implies an increase in the number of infected cells, as a result of cell-to-cell viral transmission in vivo or the proliferation of HTLV-1-infected cells themselves (2, 45). There was a mild inverse correlation between HTLV-1-specific cellular immunity and the proviral load among HTLV-1-infected rats through various routes (8), suggesting that HTLV-1-specific T-cell immunity could actively control the number of HTLV-1-infected cells in this rat model. If this hypothesis is correct, the established equilibrium set point of the HTLV-1 proviral load in an individual showing a low immune response must decrease if the HTLV-1-specific immune response is restored.

In the present study, we demonstrate that reimmunization of orally HTLV-1-infected rats with an HTLV-1-infected cell line or primary T cells results in a reduction in the HTLV-1 proviral load, indicating that HTLV-1-specific T-cell immunity is capable of controlling the number of HTLV-1-infected cells in vivo. These findings also imply that the risk of ATL may potentially be diminished by reimmunization.

MATERIALS AND METHODS

Animals. Three-week-old female F344/N Jcl-rnu/+ (F344 n/+) and ACI/NJcl rats were purchased from Clea Japan, Inc. (Tokyo, Japan). The rats were maintained at the experimental animal facilities of Tokyo Medical and Dental University and treated in accordance with the regulations and guidelines of the Animal Care Committee of the university.

Cell lines. An HTLV-1-producing human T-cell line, MT-2, and an HTLV-1-infected rat T-cell line, FPM1 (25), derived from an F344 n/+ rat were cultured in RPMI1640 medium containing 10% heat-inactivated fetal calf serum (FCS; BioWhittaker, Walkersville, MD), 100 IU of penicillin/ml, 100 μg of streptomycin/ml, and 2 mg of sodium bicarbonate/ml. G14 (36), an interleukin-2-dependent HTLV-1-negative $CD8^+$ T-cell line established from an F344 n/+ rat, and G14-Tax (36), a stable transfectant of G14 containing HTLV-1 Tax-expressing plasmids, were also used. G14 and G14-Tax cells were maintained in a RPMI 1640 medium containing 10^{-5} M 2-mercaptoethanol and 10 U of recombinant human interleukin-2 (Shionogi Pharmaceutical Co., Osaka, Japan)/ml.

Infection of rats with HTLV-1. A total of 2×10^7 to 5×10^7 MT-2 cells were treated with 50 μg of mitomycin C (MMC)/ml at 37°C for 30 min, washed, and administered to 3- to 6-week-old female rats either orally or intraperitoneally. For oral infection, MMC-treated MT-2 cells in 0.5 ml of phosphate-buffered saline were directly administered into the esophagus through a feeder tube. For intraperitoneal infection, similarly treated MT-2 cells were injected percutaneously into the abdominal cavity.

Splenectomy. A total splenectomy was performed at necropsy. A half-splenectomy was performed under anesthesia by intraperitoneal injection of ketamine (75 mg/kg) and xylazine (10 mg/kg). Splenocytes from the excised spleen halves were enriched for T cells by using a nylon-wool column and cryopreserved at −80°C. At 1 week after the operation, the rats were inoculated with 2×10^7 MMC-treated FPM1 cells. After a further 4 weeks, the rats were sacrificed, and T cells isolated from their residual spleens were cryopreserved at −80°C in the same manner as preimmunized splenocytes.

Quantification of the HTLV-1 proviral load. Genomic DNA samples (approximately 500 ng) were prepared from spleen tissue by digestion with sodium dodecyl sulfate-proteinase K, followed by phenol-chloroform extraction. The samples were then subjected to real-time PCR in a LightCycler PCR system (Roche Diagnostics, Mannheim, Germany) using Tax-specific primers, pX2 (5′-ATA CCC AGT CTA CGT GTT TGG AGA CTG T-3′) and pX3 (5′-CCG ATA ACG CGT CCA TCG ATG GGG TCC-3′), and a QuantiTect SYBR Green PCR kit (QIAGEN, Tokyo, Japan) in accordance with the manufacturer's instructions as described previously (8). The relative HTLV-1 provirus copy numbers were calculated by dividing the raw values by the amount of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) in the same sample. In some experiments, genomic DNA was also amplified by 35 cycles of PCR with the pX2/pX3 primer set, and the PCR products were directly visualized by ethidium bromide staining after 2% agarose gel electrophoresis.

T-cell proliferation assay. Rat T cells were enriched from spleen cells by passage through a nylon-wool column and used as responder cells. G14 and G14-Tax cells were treated with 1% formalin in phosphate-buffered saline for 30 min, washed, and used as stimulator cells. Responder cells (10^5 cells/well) and stimulator cells (5×10^4 cells/well) were cultured in medium containing 10% FCS in a 96-well round-bottom culture plate at 37°C for 72 h and then pulsed with [^{3}H]thymidine (37 kBq/well) for 16 h to examine T-cell proliferation. The cells were then harvested by using a Micro 96 Harvester (Skatron, Lier, Norway), and their [^{3}H]thymidine incorporations were measured in a microplate beta counter (Micro Beta Plus; Wallac, Turku, Finland). A proliferation index was calculated as the counts per minute (cpm) of the sample wells divided by the cpm of control wells containing naive splenic T cells with G14-Tax cells as stimulator cells in the same experiment.

Mixed lymphocyte reaction (MLR). Rat spleen T cells served as responder cells. Whole splenocytes from ACI rats treated with MMC were used as stimulator cells. Responder cells (2×10^5 cells/well) and various numbers of stimulator cells were cultured in RPMI 1640 medium containing 10% FCS in a 96-well round-bottom culture plate at 37°C for 5 days, and the [^{3}H]thymidine incorporation during the last 16 h of the incubation was measured.

IFN-γ production assay. Rat spleen T cells (10^5 cells/well) were cultured without or with formalin-fixed G14 or G14-Tax cells (5×10^4 cells/well) in a microtiter plate in 200 μl of medium containing 10% FCS/well for 3 days. Next, the concentrations of gamma interferon (IFN-γ) in the supernatants were measured by enzyme-linked immunosorbent assay (ELISA) using Cytoscreen Rat IFN-γ ELISA kits (BioSource International, Inc., Camarillo, CA).

Induction of a contact hypersensitivity response. Rats were sensitized and challenged to elicit a contact hypersensitivity response to 2,4-dinitrofluorobenzene (DNFB) (42, 49). The rats were sensitized by painting their shaved back with 500 μl of 1% DNFB in acetone-olive oil (4:1) on days 0 and 1. On day 6, after measurement of the ear thickness using a dial thickness gauge, each rat was challenged by applying 100 μl of 0.5% DNFB to the right side of the ear. The ear thickness was measured again at 24 h after the challenge. The extent of ear swelling was determined by the following calculation: (right ear lobe thickness at 24 h after the challenge − right ear lobe thickness before the challenge) − (left ear lobe thickness at 24 h after the challenge − left ear lobe thickness before the challenge).

Statistical analysis. Dunnett's *t* test was used for evaluating antigen-specificity in T-cell proliferation assays. A Student *t* test was used for evaluating differences between two groups of samples. *P* values of <0.05 were considered to be statistically significant.

RESULTS

HTLV-1-specific T-cell unresponsiveness in oral HTLV-1 infection. T cells from orally HTLV-1-infected rats are known to show an insufficient response to HTLV-1 antigens (22). First, we assessed whether this T-cell unresponsiveness in

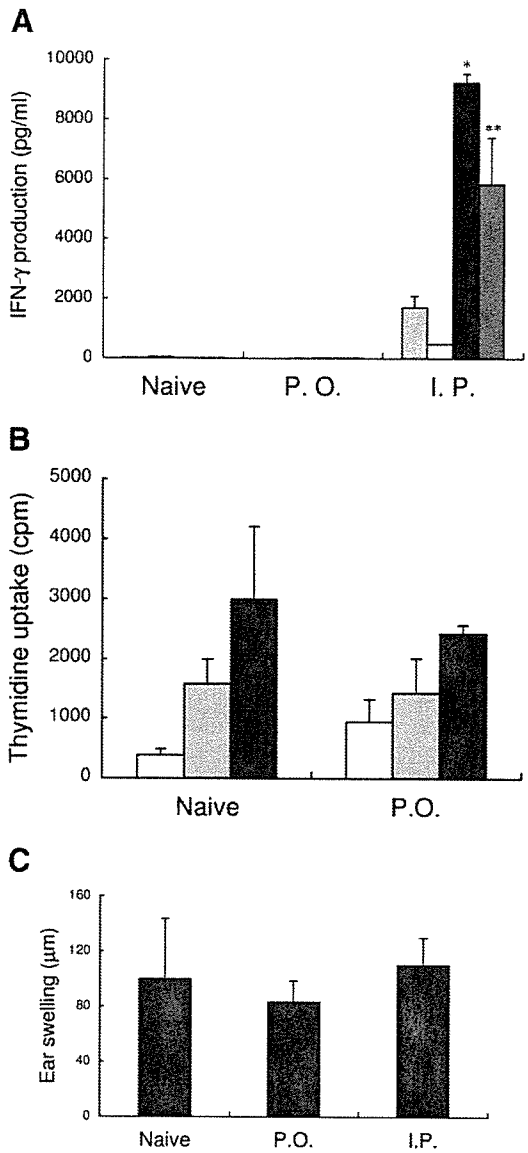

FIG. 1. HTLV-1 specificity of the T-cell unresponsiveness in orally HTLV-1-infected rats. (A) IFN-γ production in spleen T cells isolated from uninfected rats (naive) and orally (P.O.) or intraperitoneally (I.P.) HTLV-1-infected rats at 20 to 21 weeks after infection were examined by ELISA after 3 days of coculture without (▨) or with formalin-treated various syngeneic T-cell line cells, including G14 cells (□) negative for Tax, Tax-G14 cells (■) expressing Tax, and FPM1 cells (▨) infected with HTLV-1. The results represent the mean ± the standard deviation (SD). Similar results were obtained in three other sets of orally or intraperitoneally HTLV-1-infected rats. Asterisks denote statistical significance compared to values without stimulator cells: *, $P < 0.01$; **, $P < 0.05$. (B) The alloreactivities of spleen T cells (2×10^5/well) from uninfected (Naive) and orally HTLV-1-infected (P.O.) rats at 17 weeks after infection were examined by MLRs after culture without (□) or with 5×10^4/well (▨) or 1×10^5/well (■) of MMC-treated ACI rat splenocytes in a 96-well plate for 5 days and evaluated by measuring [^{3}H]thymidine incorporation during the last 16 h of culture. (C) The contact hypersensitivity responses in the skin of uninfected (Naive) and orally (P.O.) or intraperitoneally (I.P.) HTLV-1-infected rats were evaluated at 5 weeks after infection by ear swelling for 24 h after DNFB challenge, following sensitization with DNFB in their backs 1 week previously. The ear swelling was calculated as described in Materials and Methods. The results represent the mean ± the SD for three rats in each group.

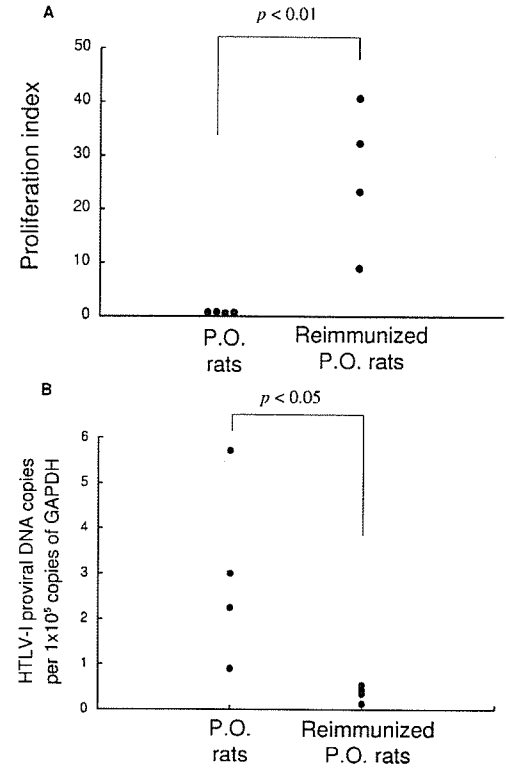

FIG. 2. HTLV-1-specific T-cell responses and proviral loads in orally infected and reimmunized rats. (A) A total of eight rats were orally infected with HTLV-1. At 7 weeks after the infection, four of the rats were left untreated (P.O. rats), while the other four rats were subcutaneously administered 2×10^7 MMC-treated HTLV-1-infected syngeneic rat FPM1 cells (Reimmunized P.O. rats). At 4 to 5 weeks after the reimmunization, T-cell-enriched spleen cells from the P.O. or reimmunized P.O. rats were subjected to proliferation assays. The proliferation index of [^{3}H]thymidine incorporation against Tax-G14 cells was calculated as described in Materials and Methods. (B) The HTLV-1 provirus loads in the spleens of the rats in panel A were measured by real-time PCR. The results represent the provirus copy numbers/10^5 copies of GAPDH.

orally HTLV-1-infected rats is specific for HTLV-1. Representative Tax-specific T-cell responses for naive, orally infected, and intraperitoneally infected rats are shown in Fig. 1A. Spleen T cells from intraperitoneally infected rats produced significant levels of IFN-γ in response to syngeneic Tax-presenting Tax-G14 or HTLV-1-infected FPM1 cells compared to those against Tax-negative G14 cells or medium controls. In contrast, IFN-γ production in orally infected rats was as low as those in uninfected rats. Similar results were obtained from all of the four sets of orally and intraperitoneally infected rats tested.

However, T cells from orally infected rats proliferated well in a set of MLR assays with allogeneic rat splenocytes (Fig. 1B). The level of T-cell proliferation in orally HTLV-1-infected rats against ACI rat spleen cells was comparable to that of naive T cells. The IFN-γ levels in the MLR supernatants were also comparable in naive and orally infected rats (data not shown).

Next, we examined the contact hypersensitivity responses, which are mainly $CD8^+$ T-cell-mediated responses at the effector phase (23), in uninfected and orally infected rats that had been sensitized by DNFB application to their backs. At 1

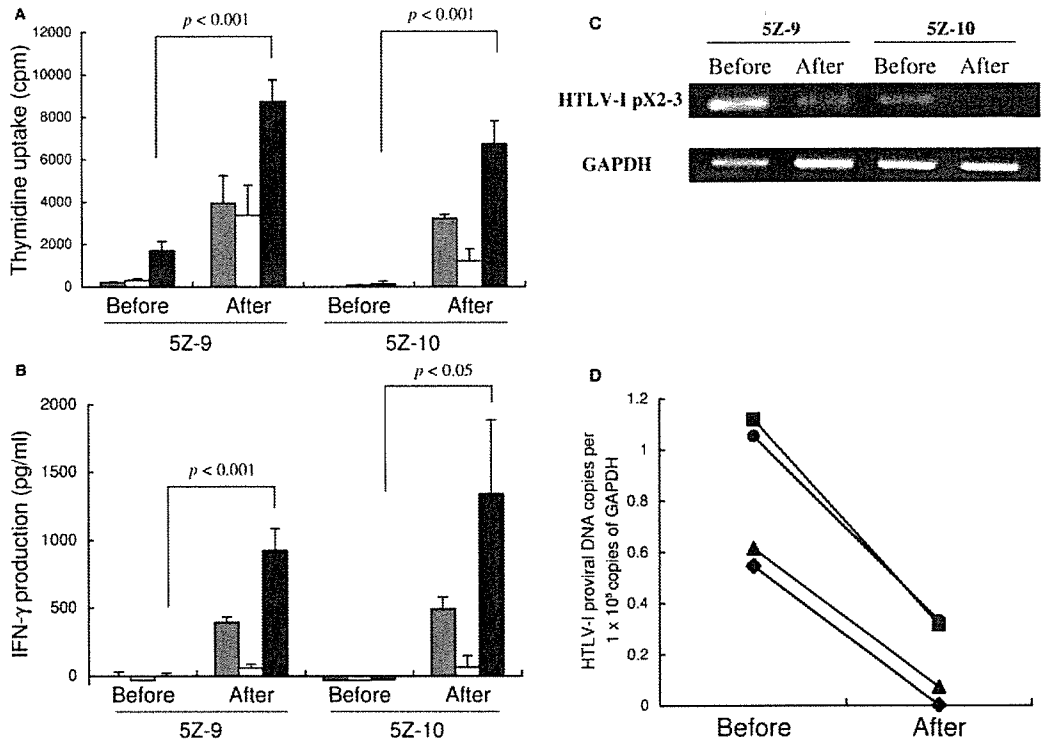

FIG. 3. HTLV-1-specific T-cell responses and proviral loads in orally infected rats before and after reimmunization. A half-splenectomy was performed in two orally HTLV-1-infected rats (5Z-9 and 5Z-10) under anesthesia at 47 weeks after infection. The T-cell-enriched fractions of the excised spleen tissues were stored at −80°C. At 1 week after the surgery, 2×10^7 MMC-treated FPM1 cells were administered subcutaneously. The residual spleens were harvested at 4 weeks after the reimmunization. (A and B) The cryopreserved spleen T-cell-enriched fractions before and after the FPM1 cell inoculation were examined for their proliferative (A) and IFN-γ production (B) responses to medium only (▨), formalin-treated G14 cells (□), or Tax-G14 cells (■) by determining the [^{3}H]thymidine incorporation and by ELISA, respectively. The results represent the mean ± the SD of triplicate wells. (C) Comparison of the amounts of HTLV-1 provirus in 5Z-9 and 5Z-10 rats before and after FPM1 cell inoculation. The spleen DNA samples were amplified by 35 cycles of PCR with Tax- and GAPDH-specific primers and visualized with ethidium bromide staining. (D) Quantification of HTLV-1 provirus loads by real-time PCR in the spleens of 5Z-9 (■), 5Z-10 (◆), and two additional orally HTLV-1-infected rats, 8H-7 (●) and 8H-9 (▲), that received a half-splenectomy at 20 weeks after infection and were immunized with MMC-treated FPM1 cells similarly to animals 5Z-9 and 5Z10.

week after the sensitization, we challenged the rats by applying DNFB to one of their ears and then measured the ear swelling at 24 h after the challenge. As shown in Fig. 1C, the orally infected rats showed levels of ear swelling similar to the uninfected rats. Thus, the T-cell responses were only insufficient against HTLV-1 and not against allogeneic or contact hypersensitivity antigens in orally HTLV-1-infected rats.

Effects of HTLV-1 reimmunization of orally HTLV-1-infected rats on HTLV-1-specific T-cell responses and the provirus load. We previously reported that HTLV-1 proviral loads are elevated in orally HTLV-1-infected rats, which may be a consequence of insufficient HTLV-1-specific T-cell responses in these rats (8). Therefore, we next examined whether these conditions in orally HTLV-1-infected rats could be altered by reimmunization with HTLV-1-infected cells. A total of eight rats were orally infected with 5×10^7 MMC-treated MT-2 cells, and then four of the eight rats were reimmunized after 7 weeks with 2×10^7 cells of the MMC-treated syngeneic HTLV-1-infected T-cell line FPM1 by subcutaneous injection. At 4 to 5 weeks after the reimmunization, the HTLV-1-specific T-cell responses and HTLV-1 proviral loads in the spleens were determined, and the results are summarized in Fig. 2A and B, respectively. The Tax-specific T-cell proliferative responses were very low in all four orally HTLV-1-infected rats that were not reimmunized. However, the reimmunized orally HTLV-1-infected rats exhibited significant levels of Tax-specific T-cell proliferation (Fig. 2A). In contrast, real-time PCR assessment of the HTLV-1 provirus loads in the rats revealed results completely opposite to the T-cell responses (Fig. 2B). Although the provirus loads in the untreated orally HTLV-1-infected rats varied among the individual rats, the reimmunized rats showed significantly lower levels of proviral load. A stronger T-cell response coincided with a lower proviral load in the reimmunized rats, suggesting that augmentation of the HTLV-1-specific T-cell response may contribute to reducing the HTLV-1 proviral load.

Reduction in the HTLV-1 provirus load after reimmunization of orally HTLV-1-infected rats. Since the levels of proviral load in the orally HTLV-1-infected rats varied among individuals, we further examined whether the established proviral load in an orally infected rat could be reduced by HTLV-1 reimmunization. In order to compare the HTLV-1-specific T-cell responses and HTLV-1 provirus loads before and after reimmunization in the same rats, we performed a half-splenectomy in two orally HTLV-1-infected rats to obtain the preimmune splenocytes and then subcutaneously reimmunized these

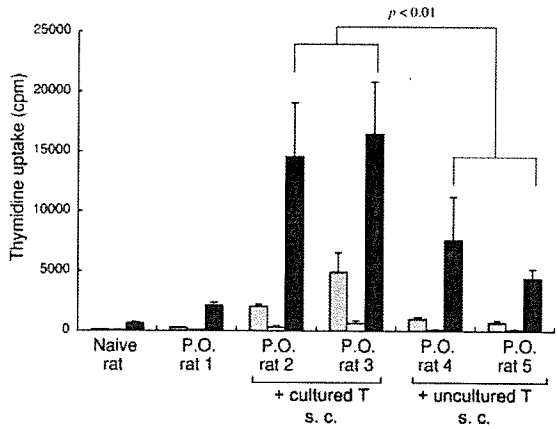

FIG. 4. Recovery of Tax-specific T-cell responses in orally HTLV-1-infected rats by subcutaneous (s.c.) inoculation with HTLV-1-infected primary T cells. Rats orally infected with HTLV-1 15 weeks previously were subcutaneously inoculated without (P.O. rat 1) or with (P.O. rats 2 to 5) primary spleen T cells (2×10^7 cells), which had been isolated from other syngeneic orally HTLV-1-infected rats and treated with MMC before the inoculation, either immediately (uncultured T) or after 2 days of culture in RPMI 1640 medium containing 10% FCS (cultured T), as indicated. After 5 weeks, the spleen T cells were harvested from these rats and subjected to proliferation assays against medium only (▨), formalin-treated G14 cells (□), or Tax-G14 cells (■). The results represent the mean [^{3}H]thymidine incorporation ± the SD in triplicate samples.

rats with MMC-treated FPM1 cells. At 4 weeks after the reimmunization with FPM1 cells, we harvested the residual spleens and examined the T-cell responses and proviral loads in the splenocytes before and after reimmunization.

The results for the two rats (5Z-9 and 5Z-10) are shown in Fig. 3. In both rats, the Tax-specific proliferative responses of the splenic T cells were very low before reimmunization but became markedly restored after reimmunization (Fig. 3A). Similar recovery of the Tax-specific T-cell responses after reimmunization in these rats was also observed in IFN-γ production assays (Fig. 3B). HTLV-1 proviruses in the spleen halves harvested from the rats before and after reimmunization were amplified by PCR using Tax-specific primers. The direct staining of PCR products indicated that HTLV-1 proviruses decreased after reimmunization in both rats (Fig. 3C). In rat 5Z-10 in particular, HTLV-1 proviruses became undetectable after reimmunization. Decreases in the provirus copy numbers after reimmunization were confirmed by real-time PCR in these spleen samples and also in spleen halves from two other orally HTLV-1-infected rats, 8H-7 and 8H-9, that were reimmunized with MMC-treated FPM1 cells after half-splenectomy, similarly to the 5Z-9 and 5Z-10 rats (Fig. 3D). These results indicate that the recovery of the T-cell response against HTLV-1 by reimmunization is directly associated with a reduction in the HTLV-1 provirus load in the host.

Recovery of the HTLV-1-specific T-cell response by subcutaneous inoculation with autologous primary T cells. The FPM1 cell line used for the reimmunization is a transformed T-cell line derived from syngeneic rat thymocytes infected with HTLV-1 in vitro, and the cells express a large amount of HTLV-1 Tax (25). However, HTLV-1-infected individuals possess HTLV-1-infected cells among their own T cells in vivo. Finally, therefore, we assessed whether subcutaneous injection of primary T cells isolated from orally HTLV-1-infected rats could abrogate the HTLV-1-specific T-cell unresponsiveness in orally HTLV-1-infected syngeneic rats.

T-cell-enriched splenocytes isolated from orally HTLV-1-infected rats were either uncultured or cultured for 2 days, treated with MMC, and subcutaneously injected into syngeneic rats that had been orally infected with HTLV-1. The T-cell responses in these rats at 5 weeks after the subcutaneous injection are shown in Fig. 4. Both of the rats injected with the MMC-treated cultured primary T cells showed significant levels of Tax-specific T-cell responses. However, the recovery of the T-cell responses in the rats injected with the uncultured primary T cells was less effective.

DISCUSSION

In the present study, we demonstrated that restoration of HTLV-1-specific T-cell immunity was associated with a reduction in the HTLV-1 proviral load in orally HTLV-1-infected rats. Together with our previous finding that orally HTLV-1-infected rats show insufficient HTLV-1-specific T-cell responses with elevated proviral loads (8), the present results strongly suggest that T-cell immunity actively controls the number of HTLV-1-infected cells in vivo.

HTLV-1 in vivo is presumably maintained by cell-to-cell transmission of the virus and multiplication of the infected cells (2, 15). HTLV-1-specific T cells potentially inhibit both pathways but only if the infected cells express target antigens. In the present study, rats were reimmunized at various periods after oral HTLV-1 infection, i.e., in the subacute and chronic phases. Although the efficiency of HTLV-1 transmission is supposed to be much lower in rats than in humans (6), there was an individual variety in the levels of proviral load established. Nevertheless, later recovery of T-cell immunity was able to reduce the viral load, indicating that the infected cells were susceptible to the immune T cells in vivo. As a result, a newly equilibrated proviral load was established.

Although the HTLV-1-specific T-cell response was markedly suppressed in the orally HTLV-1-infected rats, their T-cell responses to other antigens, such as MLR and contact hypersensitivity, were comparable to those of uninfected rats. MLR is a $CD4^+$ T-cell-dominant response to MHC II, whereas contact hypersensitivity induced by DNFB is a $CD8^+$ T-cell-mediated response to cutaneous sensitization and subsequent challenge (23). It is known that measles virus infection reduces contact hypersensitivity in a rodent model (31, 43). In healthy HTLV-1 carriers, suppressed delayed-type hypersensitivity to purified protein derivatives, as been reported in several studies (28, 33, 50), although it remains controversial (34). However, our observed T-cell unresponsiveness in orally infected rats was specific for HTLV-1 and did not merely reflect general immunosuppression. It has been suggested that transforming growth factor β and interleukin-10 produced by regulatory T cells and type 3 helper T cells are involved in oral tolerance to protein antigens (4). The precise mechanism of the HTLV-1-specific T-cell tolerance in orally infected rats remains to be determined.

It is of note that subcutaneous administration of primary spleen T cells from orally HTLV-1-infected rats induced res-

toration of HTLV-1-specific immune responses in syngeneic orally HTLV-1-infected rats. This is an apparent paradox because similar spleen T cells are already present in the hosts. This phenomenon indicates that the T-cell unresponsiveness in orally infected rats cannot be attributed to the clonal deletion of HTLV-1-specific T cells. We suppose that the HTLV-1-specific T-cell tolerance was abrogated by the subcutaneous administration of HTLV-1-infected cells via the activation of antigen-presenting cells in the skin. The use of cultured splenocytes restored the immune responses more effectively than uncultured splenocytes. This difference may be due to the amount of HTLV-1 antigens expressed in the splenocytes, since HTLV-1 expression is known to be very low in human peripheral blood and spontaneously induced during short-term culture (11, 19). Tax-induced costimulatory molecules in the infected cells may also contribute to the abrogation of immune tolerance by activating both antigen-presenting cells and T-cell responses (27, 29, 39).

In humans, HTLV-1-specific T-cell responses are exhibited by HAM/TSP patients and many asymptomatic HTLV-1 carriers. However, a small proportion of HTLV-1-carriers, including ATL patients, show repression of HTLV-1-specific immune responses. Since a high proviral load has been shown to be one of the risk factors for ATL (14, 37), the reduction in the proviral load after reimmunization demonstrated in the present study implies that restoration of HTLV-1-specific T-cell immunity potentially reduces the risk of ATL in HTLV-1 carriers with low immune responses. Autologous HTLV-1-infected cells in the peripheral blood are a potential candidate for the immunogen.

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