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

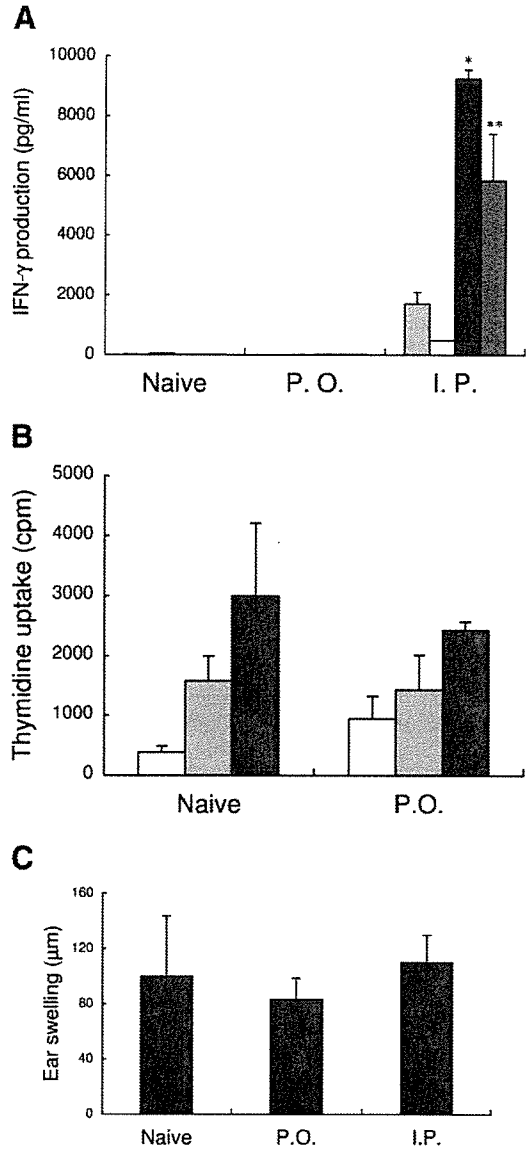

FIG. 1. HTLV-1 specificity of the T-cell unresponsiveness in orally HTLV-1-infected rats. (A) IFN-γ production in spleen T cells isolated from uninfected rats (naive) and orally (P.O.) or intraperitoneally (I.P.) HTLV-1-infected rats at 20 to 21 weeks after infection were examined by ELISA after 3 days 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.

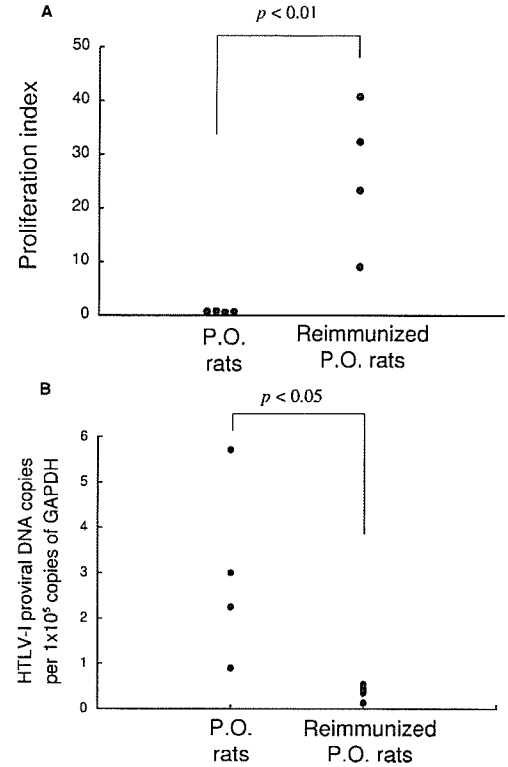

FIG. 2. HTLV-1-specific T-cell responses and proviral loads in orally infected and reimmunized rats. (A) A total of eight rats were orally infected with HTLV-1. At 7 weeks after the infection, four of the rats were left untreated (P.O. rats), while the other four rats were subcutaneously administered 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

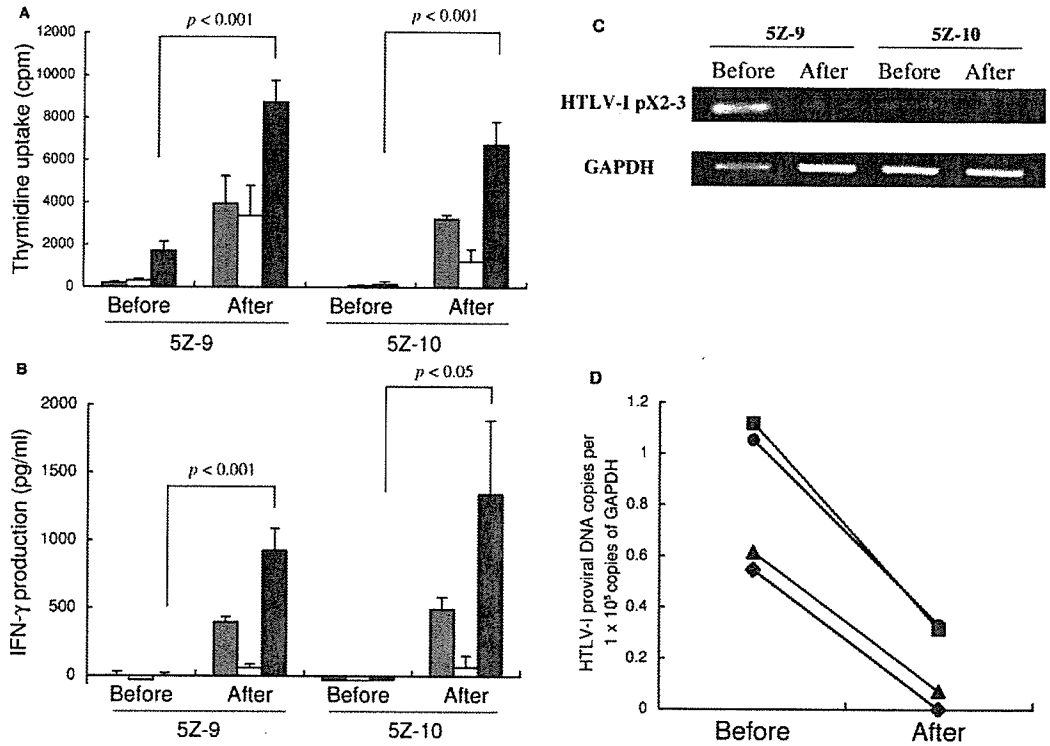

FIG. 3. HTLV-1-specific T-cell responses and proviral loads in orally infected rats before and after reimmunization. A half-splenectomy was performed in two orally HTLV-1-infected rats (5Z-9 and 5Z-10) under anesthesia at 47 weeks after infection. The T-cell-enriched fractions of the excised spleen tissues 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

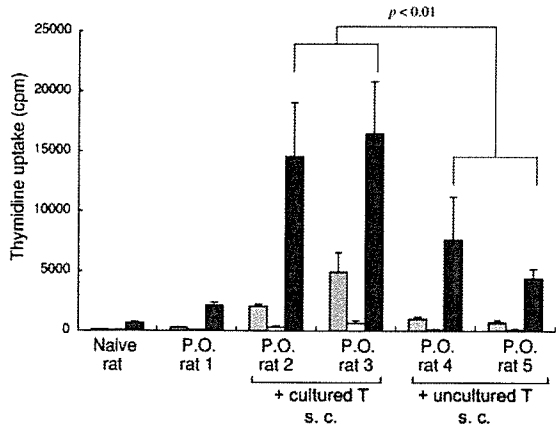

FIG. 4. Recovery of Tax-specific T-cell responses in orally HTLV-1-infected rats by subcutaneous (s.c.) inoculation with HTLV-1-infected primary T cells. Rats orally infected with HTLV-1 15 weeks previously were subcutaneously inoculated without (P.O. rat 1) or with (P.O. rats 2 to 5) primary spleen 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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抗エイズ薬開発のための小動物評価系の開発と新規治療薬の開発研究

(H16-創薬-００７)

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2／2 冊

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