Prevention of Adult T-Cell Leukemia-Like Lymphoproliferative Disease in Rats by Adoptively Transferred T Cells from a Donor Immunized with Human T-Cell Leukemia Virus Type 1 Tax-Coding DNA Vaccine

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Human T-cell leukemia virus type 1 (HTLV-1) causes adult T-cell leukemia (ATL) in infected individuals. ATL is a malignant lymphoproliferative disease affecting a subgroup of middle aged HTLV-1 carriers characterized by a low cure rate, which is mainly due to resistance to chemotherapy. Thus, establishment of an effective therapy against ATL is desirable, particularly in areas, such as Japan and Latin America, where the disease is endemic.

HTLV-1 genome contains a unique 3' region, designated pX, which encodes the viral transactivator protein, Tax (39). Tax transactivates not only the viral long terminal repeat (8, 40, 43) but also the promoters of cellular genes such as interleukin-2 (IL-2) (42), IL-2 receptor (18), myc (7), and fos (12). Thus, it is speculated that Tax plays a central role in HTLV-1-associated immortalization and transformation of T cells, which may lead to the development of ATL.

Tax is also known as a major target protein recognized by cytotoxic T lymphocytes (CTLs) of HTLV-1 carriers (21, 22). It has been reported that the levels of HTLV-1-specific CTLs are quite diverse among HTLV-1 carriers and that ATL patients have impaired levels of HTLV-1 specific CTLs in contrast to the high levels of CTL response in HTLV-1 carriers with HAM/TSP (21, 23–25, 33). Since HTLV-1 Tax-specific CTLs can recognize and lyse ATL cells in vitro, it is reasonable to assume that the low CTL activity in ATL patients is disadvantageous as it may allow uncontrolled proliferation and evolution of HTLV-1 infected cells in vivo. Therefore, stimulation of CTL response to Tax in ATL and preleukemic patients may be therapeutically beneficial and a useful prophylactic strategy against ATL.

To test this hypothesis experimentally, it is very important to use a suitable animal model system. Although several experimental trials of various treatment modalities have been reported in a variety of animal models of HTLV-1 infection (30, 31, 41), these studies did not examine the relationship between the therapeutic effects and HTLV-1-specific CTL activities. We recently established a novel rat model of ATL-like disease (32). In this model, fatal systemic lymphomas reproducibly occur in athymic F344/N Jcl-rnu/nu rats inoculated with a rat HTLV-1 infected T cell line. Vaccination with mutant Tax DNA lacking transforming ability also induced efficient anti-tumor immunity in this model. Our results indicated a promising effect for DNA vaccine with HTLV-1 Tax against HTLV-1 tumor development in vivo.

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CTL activity against Tax-expressing cells and that adoptive transfer of these CTLs effectively suppressed in vivo growth of HTLV-1-transformed tumor cells. Vaccination with mutant Tax DNA lacking transformed ability also induced efficient antitumor immunity in this model. These results suggest the potential usefulness of Tax-directed DNA vaccination against the development of HTLV-1 tumor.

MATERIALS AND METHODS

Animals. Female F344/N Jcl-mu/mu (mu/mu) rats and F344/N Jcl-mu/mu (mu/+ ) rats were purchased from Clea Japan, Inc. (Tokyo, Japan). All rats were maintained at the experimental animal facilities at Tokyo Medical and Dental University. The experimental protocol was approved by the Animal Ethics Review Committee of our University.

Cell lines. An HTLV-1-immortalized cell line, FPM1, was established in our laboratory by cocultivating thymocytes of mu/+ rat with the HTLV-1-producing human cell line, MT-2, which was treated with mitomycin C (50 μg/ml) for 30 min at 37°C (26). The cells were maintained in RPMI 1640 with 10% heat-inactivated fetal calf serum (FCS) (Whittaker, Walkersville, Md.), penicillin, and streptomycin. IL-2 (10 U/ml; Shionogi, Osaka, Japan) was added at the beginning of coculture. Cells were eventually freed from exogenous IL-2. FPM1-WT subcloned from FPM1 cells, which proliferates in vivo growth in mu/mu rats (32). Another HTLV-1-immortalized rat T-cell line, derived from a WKA rat, TAR3-1 (46), was kindly provided by Takashi Yoshiki (Hokkaido University School of Medicine, Sapporo, Japan).

HTLV-1-negative simian virus 40 (SV40)-transformed rat kidney cell line (FPM-SV) was established in our laboratory from kidney cells of a mu/+ rat (26). Briefly, kidney cells cultured for 1 week were infected with SV40 at 37°C for 1 h and then washed and cultured for 3 weeks with replacement of culture medium twice a week. A focus growing in the culture was picked up and sequentially expanded to a stable line.

Establishment of G14 and G14-Tax cell lines. An IL-2-dependent HTLV-1-negative rat cell line, G14, was established from a mu/+ rat initially for the purpose of obtaining FPM1-specific CTLs. Briefly, nylon wool column-purified splenic T cells of an HTLV-1-infected mu/+ rat were stimulated in vitro with formalin-fixed FPM1 cells twice in a month in the presence of IL-2. These T cells maintained FPM1-specific cytotoxic activities for the first 3 months of culture and then started to lose the specific activities. After 4 months, these cells became capable of continuously growing in a medium containing 10 U of IL-2 per ml in the absence of FPM1 for stimulation. We designated these cells as the G14 cell line. This cell line was positive for rat CD5, CD8, CD25, MHC-I, and MHC-II antigens.

To establish G14-Tax cells, G14 cells were electroporated with Tax-expressing plasmids by using Gene Pulser II systems (Bio-Rad, Hercules, Calif.), and stable transfectants were selected with 400 μg/ml of G418. MT-2 cells, a derivative of the wild-type Tax expression vector expresses the full-length of the wild-type Tax protein, whereas Tax140 mutant has two amino-acid substitutions of Gln/Glu to Ala/Sec at positions 310 and 311. Fifty milligrams of Au particles (radius, 1.6 μm; Bio-Rad) were coated with 100 μg of the expression vectors. The DNA-coated Au particles were introduced in Tefzel tubing (Bio-Rad) placed on a Tuing Prep Station and dried by rotation of the tubing under nitrogen flow (0.3 to 0.4 ml/min) for 15 min. The tubing was then cut into 12-mm-long cartridges, which were used in the Helios Gene Gun system (Bio-Rad).

Inoculation of DNA in vivo. The Helios Gene Gun system was used for the inoculation of the expression vectors. mu/+ rats were anesthetized with ketamine, and their fur was completely removed by using a commercial depilatory agent. DNA-coated Au particles in a cartridge were accelerated by pressurized helium gas for penetration through cell membranes and multiple layers of cells in the epidermis. The concentrations of DNA and Au were 1 μg/μl and 0.5 μg/μl, respectively. Immunization was performed twice, with a 1-week interval, and 10 shots were given per immunization.

Protection assay. G14 or G14-Tax cells were reseeded in an ice-cold extraction buffer (20 mmol of HEPES [pH 7.9] per liter, 10 mmol of KC1 per liter, 1 mmol of MgCl2 per liter, 150 mmol of NaCl per liter, 1% Triton X-100, 0.5 mmol of diethiothreitol per liter, 0.5 mmol of phenylmethylsulfonyl fluoride per liter, 1 μg of aprotinin per ml, and 1 μg of leupeptin per ml) and gently rocked for 30 min. After centrifugation at 14,000 × g for 20 min at 4°C, the supernatant was collected as a whole-cell extract. The protein concentration of each sample was determined using a protein assay kit (Bio-Rad). Then, 50 μg of the whole-cell extract was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12.5% gel and transferred to a nitrocellulose filter. After incubation with blocking buffer (2% bovine serum albumin in 10 mmol of Tris-HCl [pH 7.5] and 100 mmol of NaCl per liter), the filter was incubated with 1:1,000-diluted sera containing anti-Tax antibody and then with an anti-human immunoglobulin antibody conjugated to horseradish peroxidase (Amersham, Arlington Heights, Ill.). Antibody binding was detected by the enhanced chemiluminescence method (Amersham).

Cr-release cytotoxicity assay. CTL activity against Tax-expressing or HTLV-1-infected cells was measured by 51Cr-release assay at various effector/target (E/T) ratios as described previously (3). Splenocytes from each immunized rat were passed through a nylon wool column, cocultured with formalin-fixed FPM1-V1AX cells for a week, and then used as effector cells. 51Cr-labeled FPM1-V1AX or FPM1-SV cells (104 G14-Tax or G14 cells were used as HTLV-1-infected and Tax-expressing target cells, respectively. The 51Cr-labeled target cells (104/well) were cocultured with various numbers of effector cells in 96-well U-bottom culture plates at 37°C for 6 h, and then the 51Cr activities released in the supernatants were measured. Specific cytotoxicity was calculated as follows: [(experimental 51Cr release – spontaneous 51Cr release)/(maximum 51Cr release – spontaneous 51Cr release)] × 100%.

Generation of HTLV-1-specific CTL cell lines. For induction of HTLV-1-specific CTLs in long-term cultivation, splenic T cells (2.5 × 106 cell/well) were cocultured with the same number of formalin-fixed FPM1-V1AX cells in 24-well flat-bottom culture plates in RPMI 1640 medium with 10% FCS and 20 U of IL-2 per ml, with periodic stimulation using formalin-fixed FPM1-V1AX cells every 2 weeks. These cells that maintained HTLV-1-specific CTL activities for more than 3 months were judged as the CTL lines and were used in the experiments.

T-cell proliferation assay. Splenic T cells from immunized rats were purified through a nylon wool column (104 cells/well) and were cocultured with formalin-fixed FPM1-V1AX (G14, Tax-expressing) or P3-161, G14 cells (5 × 104 cells/well; 10:1 E/T) for 72 h. Cultures were pulsed with [3H]thymidine ([3H]TdR; 37 kBq/well) for the last 18 h to assess cell proliferation. Cells were harvested with a Micro 96 Harvester (Skatron, Lier, Norway), and [3H]TdR uptake into cells (reported as mean counts per minute, the standard deviation [SD]) was measured in a microplate β counter (Micro Beta Plus; Wallac, Turku, Finland).

Adoptive transfer of splenic T cells into nude rats. Two weeks after primary immunization and one week after booster immunization, 104 freshly isolated T-cell-enriched splenocytes from vaccinated rats were intraperitoneally inoculated into 4-week-old nu/nu rats, which were simultaneously inoculated subcutaneously with 2 × 107 FPM1-V1AX cells. nu/nu rats inoculated with FPM1-V1AX alone or with splenocytes from age-matched mu/+ rats inoculated with plasmids served as controls. The size of each subcutaneous tumor was measured every other day.

Measurement of growth of subcutaneously inoculated HTLV-1-immortalized cells. The growth of subcutaneous tumor was measured every other day and recorded as the longest surface length (a [in millimeters]) and width (b [in millimeters]). Tumor volume (V [in cubic millimeters]) was calculated according to the following formula: V = a × b2/2 × 0.5, as described previously (32).

RESULTS

DNA vaccine with Tax induces CTL responses specific to Tax without the production of Tax antibody. We first investigated whether DNA vaccination with Tax was capable of inducing specific CTL activity against Tax. For the analysis of Tax-specific CTL activities, we introduced Tax expression vectors into the HTLV-1-negative G14 cell line and established G14-Tax cells, which expressed detectable levels of Tax protein (Fig. 1A).

As a Tax-coding DNA, we used pβMT-2Tax plasmids which contained the entire length of wild-type Tax cDNA driven by β-actin promoter. For vaccination, gold particles coated with the plasmids were shot by a gene gun into the skin of mu/+ rats twice with a 1-week interval. One week after the second immunization, spleen T cells isolated from a vaccinated rat were restimulated in vitro for a week with the HTLV-1-infected cell line, FPM1-V1AX, and then were subjected to 51Cr release CTL assay. The representative result of four individual experiments is shown in Fig. 1B. Spleen T cells from rats immunized with Tax plasmids specifically recognized and killed G14-Tax cells but not parent G14 cells. In contrast, spleen T cells from rats inoculated with control pHBPr:1-neo plasmids did not show CTL activity against G14-Tax cells. These results indicated that DNA vaccine with Tax effectively induced Tax-specific CTLs in vivo. On the other hand, sera from rats with Tax-coding DNA vaccine did not contain any detectable levels of antibodies specific to Tax during the period tested when analyzed by Western blotting (data not shown).
Tax-specific CTLs induced by DNA vaccine specifically lyse HTLV-1-infected cells. We next examined whether these Tax-specific CTLs can lyse HTLV-1-infected cells. HTLV-1-infected FPM1-V1AX cells and HTLV-1-negative FPM-SV cells served as targets of CTL assays. Figure 2A shows the representative result of three independent experiments. Splenocytes from a rat inoculated with the control pHβPr.1-neo vector did not show CTL activity against FPM1-V1AX or FPM-SV cells. On the other hand, splenic T cells from a rat immunized with pHβMT-2Tax showed a strong cytotoxic activity against FPM1-V1AX cells but not against FPM-SV cells. MHC restriction of the Tax-specific cytotoxicity was further investigated by using spleen T cells from rats immunized with pHβMT-2Tax, which were cultured with periodic stimulation using formalin-fixed FPM1-V1AX cells every 2 weeks for 3 months. Figure 2B shows a representative result of three individual experiments. These cells significantly lysed syngeneic FPM1-V1AX cells but not the allogenic HTLV-1-infected cell line, TARS-1. A control CD8+ T-cell line, G14, did not show detectable levels of CTL activity against FPM1-V1AX or TARS-1 cells. These results indicated that the vaccine-induced CTLs were able to specifically kill syngeneic HTLV-1-infected cells.

Induction of HTLV-1-specific proliferative responses by Tax-coding DNA vaccine. To confirm that the Tax-coding DNA vaccine induces HTLV-1-specific T-cell immunity in the hosts, we examined T-cell proliferative responses against HTLV-1 antigens in rats inoculated with the DNA vaccine. We used HTLV-1-infected FPM1-V1AX cells, Tax-expressing G14-Tax cells, and HTLV-1-negative G14 cells for stimulator cells of a proliferation assay. T-cell-enriched spleen cells from Tax-coding DNA vaccine or control plasmid inoculated rats were incubated in the presence or absence of formalin-fixed stimulator cells, and thymidine incorporation in the splenic T cells was measured. As shown in Fig. 3, spleen T cells from rats inoculated with control plasmid hardly proliferated in response to any stimulator cells used. In contrast, spleen T cells from Tax-coding DNA vaccine or control plasmid inoculated rats were incubated in the presence or absence of formalin-fixed stimulator cells, and thymidine incorporation in the splenic T cells was measured. As shown in Fig. 3, spleen T cells from rats inoculated with control plasmid hardly proliferated in response to any stimulator cells used. In contrast, spleen T cells from Tax-coding DNA vaccine or control plasmid inoculated rats showed significant levels of proliferative response against FPM1-V1AX cells. This proliferative response was specific to Tax, since these splenic T cells also respond to Tax-expressing G14-Tax cells but not to parental G14 cells.

T cells induced by Tax-coding DNA vaccine inhibit the growth of HTLV-1-infected cells in vivo. We further examined whether splenocytes from Tax-immunized rats have protective activities against the growth of HTLV-1-infected lymphomas in vivo. Freshly isolated spleen T cells from rats immunized with Tax
plasmids or control pHbPr.1-neo vectors were intraperitoneally transferred into each group of six nu/nu rats at the time of subcutaneous inoculation of FPM1-V1AX cells. Tumor growth was evaluated by measuring the size of subcutaneous tumors. As shown in Fig. 4, significant suppression of tumor growth was observed in rats treated with Tax-DNA-immunized T cells in the first week of FPM1-V1AX inoculation, compared with other groups of rats that were untreated or treated with control vector-immunized T cells. After 10 days, tumors showed a complete regression in rats treated with Tax-immunized T cells (Fig. 5a). During the same period, tumor regression was also noted in a lesser degree in rats treated with control vector-immunized T cells (Fig. 5b), probably because of the delayed induction of the tumor-specific T cells from reconstituted T cells in nude rats after the transfer of naive T cells. In contrast, subcutaneous tumors continued to grow in untreated rats (Fig. 5c).

At autopsy, there were no metastatic lesions in rats treated with Tax-DNA-immunized T cells (Fig. 5d), whereas two of six rats treated with control pHbPr.1-neo vector-immunized T cells had metastasis in the lymph nodes. One of these rats also showed metastasis in the lung (Fig. 5e). In untreated rats, metastases were consistently present in the lungs (Fig. 5f), liver, and lymph nodes. Thus, adoptively transferred T cells induced by Tax-coding DNA vaccination inhibited ATL-like lymphoproliferative disease more promptly and efficiently in vivo than did control T cells.

Protective effects on the growth of HTLV-1-infected cells by DNA vaccine with mutant Tax are equivalent to those by wild-type Tax. Although Tax DNA vaccination induce HTLV-1-specific T-cell responses effective to suppress in vivo growth of HTLV-1 tumor cells, wild-type Tax is not suitable for clinical use because of its potential oncogenicity. In the next series of experiments, we assessed the antitumor effects of mutant Tax DNA vaccination in the same animal model. Mutant Tax410, which lacks transforming activities, induced the expression of Tax protein in rat fibroblasts, similar to wild-type Tax (data not shown). We then examined the ability of spleen T cells from rats vaccinated with DNA coding the wild-type Tax or Tax410 to induce specific CTL activities against HTLV-1-infected cells. As shown in Fig. 5a, DNA vaccine with Tax410 induced almost equivalent levels of CTL activity against HTLV-1-infected FPM1-V1AX cells to those of wild-type Tax-immunized rats. T cells from rats immunized with the mutant Tax also showed a significant level of inhibitory activity in vivo against the growth of HTLV-1-infected lymphoma cells in nu/nu rats (Fig. 5b), indicating that similar vaccine effects were induced by Tax410.

**DISCUSSION**

Our study demonstrated that HTLV-1 Tax-coding DNA vaccination induced Tax-specific T-cell proliferative and CTL responses and that the vaccine-induced T cells were capable of suppressing the growth of HTLV-1-infected tumor cells in vivo. These results suggest that HTLV-1 Tax served as a tumor-specific transplantation antigen in HTLV-1-infected lymphoproliferative disease. This is in agreement with previous observations in the human system in vitro that Tax is a major target antigen of HTLV-1 specific CTLs (21, 22), that this CTL activity is poorly detectable in ATL patients (23, 24), and that ATL cells are susceptible to these CTLs in vitro (23, 24).

Although DNA administered by a gene gun tends to induce Th2-cell and B-cell responses (4, 9, 14, 35), several studies have demonstrated the induction of CTL responses against a variety of pathogens, including influenza virus (11, 20), lymphocytic choriomeningitis virus (50), *Listeria* sp. (10), HIV (16), and Sendai virus (5). Under the experimental conditions used in our study, delivery of the Tax-coding DNA by the gene gun induced Tax-specific CTLs but not antibody responses during the 2 weeks after vaccination. We continued to check the production of Tax-specific antibodies in the DNA vaccine-inoculated rats for 3 months but failed to detect them. Furthermore, Tax-specific antibodies were not detected in the sera of nu/nu rats subjected to the adoptive transfer of immune T cells (data not shown). These results indicate that Tax-specific T-cell immunity but not antibodies was critical for the rejection of HTLV-1 tumor cells. This conclusion is further strengthened by our recent findings that inhibition of T-cell activation by in vivo treatment with anti-CD80 and anti-CD86 antibodies allowed the growth of HTLV-1 tumors in rats (15).
Rats have been used in studies of HTLV-1 because they are susceptible to the virus and because the virus-transformed T-cell lines can be established in rats in vitro (19, 46). Moreover, CTLs specific to HTLV-1 Gag, Env, and PX proteins could be induced in various strains of rats infected with HTLV-1 (44, 45). Although these CTLs have been shown to exhibit cytolytic activities against cells expressing corresponding antigens, whether they are able to inhibit the growth of HTLV-1 infected cells in vivo has not yet been determined. In this regard, we have recently shown in a rat model system the importance of HTLV-1-specific T cells in inhibiting the growth of HTLV-1-infected tumor cells in vivo (32). Furthermore, our present results clearly showed that Tax-specific CTLs induced by the gene gun vaccination were able to inhibit the growth of HTLV-1-infected tumor cells in vivo. These results suggest that DNA vaccines with Tax are potentially useful for the treatment of ATL and that our rat model is suitable for further investigation of the possible application of the vaccine for ATL.

There is ample evidence to suggest that the expression of viral proteins is repressed in vivo in HTLV-1 carriers (23). However, the extent and exact site of in vivo HTLV-1-infected cells expressing HTLV-1 antigens remain controversial. The HTLV-1-infected FPM1-V1AX cells used in our study predominantly express Tax proteins in vitro, although the expression of other HTLV-1 structural proteins are repressed (26). Thus, it is possible that the protective effects of Tax-specific CTLs may contribute to the inhibition of HTLV-1-infected tumor cell growth in vivo.
CTLs were easily detected in our model compared to ATL patients. Nevertheless, we could still expect that the Tax-specific CTLs would be effective against HTLV-1-infected cells during the course of development of ATL, because Tax-specific CTLs have actually been detected in HTLV-1 carriers (25, 33), indicating that Tax is actually expressed in HTLV-1-infected cells at a certain stage of HTLV-1 infection.

Tax is thought to be a critical factor in leukemogenesis because of its transforming activity in various experimental systems (49). This means that the inoculation of Tax expressing vectors may induce inappropriate effects which lead to the transformation of normal cells in vivo. To avoid this adverse effect, we examined the effects of vaccines prepared using Tax410 mutant, which lacks transforming activities. Our results demonstrated that the immune responses induced by Tax410 mutant were almost identical to those induced by wild-type Tax, indicating that Tax410 is a safer agent to induce effective immune response against HTLV-1 tumor. This finding also suggests that the transactivation effect of Tax, which can induce a number of cellular genes associated with immune responses, such as IL-2, IL-2 receptor, and IL-6 (18, 29, 42), is not related to the effective induction of Tax-specific CTLs by gene gun application.

In conclusion, we demonstrated in the present study that adoptively transferred T cells induced by Tax-coding DNA vaccine prevented the development of experimentally induced ATL-like lymphoproliferative disease in rats. Furthermore, we also demonstrated that mutant Tax, which lacks transforming activities, also induced efficient antitumor activities in vivo. These findings provide important implications of safe and effective vaccine design for the prophylaxis and treatment of ATL.

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