Summary

Human T lymphocyte virus type I (HTLV-I) can be transmitted into several inbred strains of newborn and adult rats by inoculating newly established HTLV-I-immortalized rat T cell lines or the human T cell line MT-2. The transmission efficiency exceeds 80%, regardless of strain differences or the age at transmission. The production of anti-HTLV-I antibodies significantly differs among the strains and depends on the age at the time of transmission. Rats neonatally inoculated with HTLV-I-positive rat or human cells generally become seronegative HTLV-I carriers throughout their lives, whereas adult rats inoculated with HTLV-I-positive cells at 16 wk of age become seropositive HTLV-I carriers. The HTLV-I provirus genome is present in almost all organs, regardless of whether the carriers are seronegative or seropositive. According to antibody titers to HTLV-I, there are three groups of inbred rat strains: ACI, F344, and SDJ (high responders); WKA, BUF, and LEJ (intermediate responders); and LEW (low responder). Three of three 16-mo-old seronegative HTLV-I carrier rats of the WKA strain developed spastic paraparesis of the hind legs. Neuropathological examinations revealed that the lesions were confined primarily to the lateral and anterior funiculi of the spinal cord. Both myelin and axons were extensively damaged in a symmetrical fashion, and infiltration with massive foamy macrophages was evident. The most severe lesions were at levels of the thoracic cord and continued from the cervical to the lumbar area. These histopathological features as well as clinical symptoms largely paralleled findings in humans with HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP). These HTLV-I carrier rats, in particular the WKA rats described above, can serve as a useful animal model for investigating virus-host interactions in the etiopathogenesis of HTLV-I-related immunological diseases, particularly HAM/TSP.
of the HTLV-I provirus genome were investigated. Development of HAM/TSP-like myelopathy in HTLV-I carrier rats was also monitored. This is the first description of an animal model for the HAM/TSP that occurs in humans.

Materials and Methods

Cell Lines. Four HTLV-I-immortalized rat cell lines, Lewis-S1, WKA-S1, F344-S1, and ACI-S1, were newly established by cocultivating spleen cells of four inbred rats of LEW/Hkm(LEW), WKAH/Hkm(WKA), F344/SIc(F344), and ACI/Hkm(ACI) strains with ATL cells from a typical ATL patient after treating them with 5-bromo-2'-deoxyuridine (BrdUrd), as described elsewhere (17). The cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS, penicillin(100 IU/ml), and streptomycin(100 μg/ml), and were free from exogenous IL-2.

The HTLV-I producer human cell line, MT-2, was kindly provided by Dr. I. Miyoshi (Department of Medicine, Kochi Medical College, Kochi, Japan) (13).

Antibodies. RTH-7, R1-3B3, and R1-10B5 were mAbs that reacted with the rat homologues of CD4, CD5, and CD8, respectively, and were generously provided by Drs. A. Matsuura and K. Kikuchi (Sapporo Medical College, Sapporo, Japan) (21, 22). Anti-Leu-3a (Becton Dickinson & Co., Mountain View, CA) and ART 18 (23) (Boehringer Mannheim, Mannheim, Germany) were the mAbs used against human CD4 and rat IL-2 receptor, respectively. Polyclonal antibodies against rat and human Ig were purchased from Organon Teknika Co. (Durham, NC).

Animals and Transmission of HTLV-I. Inbred female WKA, LEW, ACI, SDJ/Hkm(SDJ), BUF/Hkm(BUF), and LEJ/Hkm(LEJ) rats were obtained from the Institute for Animal Experiment (Hokkaido University School of Medicine, Sapporo, Japan). Inbred female F344 rats were purchased from SLC Japan (Shizuoka, Japan). 10^7 Lewis-S1 and WKA-S1 cells, which were treated with Mitomycin C (MMC; Kyowa Hakko, Co., Tokyo, Japan), at a concentration of 25 mg/ml for 30 min at 37°C, were inoculated into the peritoneal cavity of newborn LEW and WKA rats within 24 h after birth, respectively. 10^7 MT-2 cells were inoculated into the peritoneal cavity of newborn LEW, WKA, F344, and ACI rats within 24 h after birth. 10^7 MT-2 cells were injected into the tail vein of 16-wk-old LEW, WKA, F344, ACI, SDJ, BUF, and LEJ rats twice, at 2-wk intervals. HTLV-I-infected rats were maintained under the P3 level.

To identify the anti-HTLV-I antibody and HTLV-I provirus genome, peripheral blood was taken from the tail vein.

Detection of Antibodies against HTLV-I Antigens. The titer of antibody against HTLV-I antigens in the serum and cerebrospinal fluid (CSF) was determined by the particle agglutination test (Serodia·HTLV-I, Fuji Rebio Inc., Tokyo, Japan).

Polymerase Chain Reaction. DNA was isolated from Ficoll-Paque separated PBMC or organ tissues using SDS and proteinase K methods (24). PCR was carried out as described (25). Briefly, 0.5 μg of extracted DNA was subjected to 35 cycles of PCR amplification using Taq polymerase (Takara Shuzo Co., Kyoto, Japan) at 94°C for 1 min, 58°C for 2 min, and 72°C for 3 min. The primers were 5'-2328 GCCAAACCCAAGATCACTTTAAGC 2351-3' (sense) and 3'-2588 GGAATTCTTCTTAGCGGAG 2607-5' (antisense) for pol region amplification (Synthetic Genetics Inc., San Diego, CA), and 5'-7358 CCGATACCCAGTCTACGTGT 7377-3' (sense) and 3'-7516 GCTACCCTGCGCAATAGCGGAG 7496-5' (antisense) for pX region amplification (26). Each PCR product was subjected to electrophoresis on a 2% agarose gel and transferred to nylon membrane. The membrane was hybridized with radioactively labeled oligonucleotide probes: 3'-2498 GTTGTCCGGCCACATAGCAGCGGTCTGCGGT 2537-5' (antisense) for pol region and 3'-7447 AGGTGATCTGATGCTCTGGACAGGGTGCGTAGGCGGCG 7486-5' (antisense) for pX region. The membrane was washed in 2x SSC, 0.2% SDS at room temperature for 15 min, twice, and in 0.2x SSC, 0.2% SDS at 55°C for 15 min, twice. The specific amplified bands were detected by exposure to Kodak XAR film at −70°C. For the standard control, the detection limit of the PCR was up to 5^{-8}.

Light Microscopy. In addition to routine hematoxylin and eosin (HE) stain, Luxol fast blue (LFB), Bodian, and Holzer stains were used.

Results

Characterization of Newly Established HTLV-I-immortalized Rat T Cell Lines. In four newly established HTLV-I-immortalized rat cell lines, it was confirmed that at least one full length of HTLV-I provirus genome was integrated, tran-
scribed, and expressed, using Southern, Northern, and Western Blots analyses, respectively. Analyses of cell surface markers showed these cell lines to be positive for rat CD4 and CD5, and to be negative for rat CD8 and human markers. They also expressed the rat IL-2 receptor. The results are summarized in Table 1. Morphologically, nuclear polymorphism resembling ATL cells was seen in a large proportion of WKA-S1 and ACI-S1 cells. The other cell lines also contained cells with nuclear polymorphism, albeit to a lesser extent. Only in the ACI-S1 cell line were extracellular type C virus particles sometimes evident, as seen electron microscopically.

Infectious Transmission of HTLV-I into Newborn and Adult Rats. Lewis-S1 and WKA-S1 cells were injected into newborn LEW and WKA rats. Purified DNA from PBMC was subjected to PCR amplification, using both pol and pX primers. Samples in which PCR results were positive for both pol and pX primers were considered to be positive. The HTLV-I provirus genome was evident in PBMC from 16 of 18 LEW rats, and all of three WKA rats at 4-6 mo after birth (Table 2). We considered that Lewis-S1 and WKA-S1 cells produced HTLV-I virus and had infectivity potential for the newborn rats, however, antibodies against HTLV-I were never detected in these rats at 1-7 mo after birth. When MT-2 cells were injected into newborn LEW, WKA, F344, and ACI rats, the HTLV-I provirus genome was detected in PBMC from all of eight LEW rats (100%), eight of nine WKA rats (89%), 16 of 20 F344 rats (80%), and seven of eight ACI rats (88%). Only 2 of 16 F344 rats seroconverted for anti-HTLV-I antibodies, the titers being between 1:8 and 1:16. None of the other rats had anti-HTLV-I antibodies (Table 2).

When MT-2 cells were injected intravenously into seven different strains of adult rats, all five (100%) in each strain (except for F344, since only one rat was tested) carried the HTLV-I provirus genome in their DNA extracted from PBMCs 4 mo after injection (Table 3). Anti-HTLV-I antibodies were detected in all these rats as early as 3 wk after injection, and the titers ranged between 1:8 and 1:8,192 thereafter. The specificity of these antibodies against HTLV-I antigen was confirmed by evidence of HTLV-I-specific gag proteins (p19, p24, p53), obtained using Western Blots (data not shown).

Humoral Immune Response against HTLV-I. Humoral antibody responses against HTLV-I were compared. Adult LEW, WKA, F344, ACI, SDJ, BUF, and LEJ rats were given intravenous injections of $10^7$ MT-2 cells, twice, as described above, and anti-HTLV-I antibodies in the sera were measured at 2, 3, 7, 13, 21, 28, 40, and 49 wk after the first injection. As shown in Fig. 1, the antibodies were detectable as early as 2-3 weeks after the injection, and a plateau was reached at 12 wk. Antibody titters tended to decline slightly after 28 wk but were high at 49 wk. ACI and SDJ rats were considered to be high responders, as the titer of anti-HTLV-I antibodies was higher than for other strains, and LEW rats were low responders, with low anti-HTLV-I antibodies. F344 rats produced a high level of anti-HTLV-I antibodies for up to 13 wk after the first injection. Further measurement of anti-HTLV-I antibody was done in only one of F344 rats maintaining a high level of antibody titer of 1:4,096 at 28 wk. The F344 strain was thus considered to be a high responder, like the ACI and SDJ strains. Rats of the other three strains produced anti-HTLV-I antibodies with intermediate titers, and were classed as intermediate responders. The difference in humoral response against HTLV-I is statistically significant (student's t test).

Tissue Distribution of the HTLV-I Provirus Genome. Five female F344 rats injected with MT-2 cells in the neonatal period were killed at 50 wk. DNAs extracted from PBMC and tissues from each organ were subjected to PCR amplification, using pX primers. Each DNA sample was PCR analyzed more than twice, and the sample in which the HTLV-I provirus genome was reproducibly demonstrated was judged as positive. The HTLV-I provirus genome was detected in almost all organs examined, including the thymus (5/5), kidney (5/5), spleen (4/5), submandibular gland (4/5), lymph nodes (5/5), and PBMC (5/5) (Table 4). The PCR result is shown in Fig. 2.

Development of HAM/TSP-like Myelopathy. Eight of nine WKA rats neonatally inoculated with MT-2 cells became HTLV-I carriers yet had no detectable anti-HTLV-I antibodies.

| Table 2. Transmission of HTLV-I into Newborn Rats |
|-----------------------------------------------|
| Strain | Rat MHC | Cells injected (10^7) | Anti-HTLV-I antibodies* | Presence of HTLV-I provirus genome† |
|--------|---------|----------------------|------------------------|-----------------------------------|
| LEW 1  | RT1 18  | LEW-S1               | 0/18 (0%)              | 16/18 (89%)                       |
| WKA k  | 3       | WKA-S1               | 0/3 (0%)               | 3/3 (100%)                        |
| LEW 1  | 8       | MT-2                 | 0/8 (0%)               | 8/8 (100%)                        |
| WKA k  | 9       | MT-2                 | 0/9 (0%)               | 8/9 (89%)                         |
| F344 lv1| 20      | MT-2                 | 2/20 (10%)             | 16/20 (80%)                       |
| ACI av1| 8       | MT-2                 | 0/8 (0%)               | 7/8 (88%)                         |

* Anti-HTLV-I antibodies were measured at 1 and 7 mo after birth.
† Presence of the HTLV-I provirus genome was determined 4-6 mo after birth.
Table 3. Transmission of HTLV-I into Adult Rats

| Strain | Rat MHC | Age at first MT-2 injection* | Anti-HTLV-I antibodies | Presence of HTLV-I provirus genome† |
|--------|---------|-----------------------------|-------------------------|-----------------------------------|
| LEW    | l       | 5 wk                        | 5/5                     | 5/5                               |
| WKA    | k       | 5 wk                        | 5/5                     | 5/5                               |
| F344   | lv1     | 5 wk                        | 5/5                     | 5/5                               |
| ACI    | av1     | 5 wk                        | 5/5                     | 5/5                               |
| SDJ    | u       | 5 wk                        | 5/5                     | 5/5                               |
| BUF    | b       | 5 wk                        | 5/5                     | 5/5                               |
| LEJ    | j       | 5 wk                        | 5/5                     | 5/5                               |

* 10⁷ cells of MT-2 were injected into the tail vein twice at 2-wk intervals.
† Purified DNA from PBMC was subjected to PCR amplification at 4 mo after injection.

(Table 2). Four rats were killed at 4 mo, and tissue distribution of provirus genomes was examined. One of the remaining four male rats died at 12 mo of unknown causes and an autopsy was not done. At 16 mo, two of three rats were first seen to have spastic paraparesis of the hind legs, and 2 wk later, the remaining had similar symptoms. The spastic paraparesis progressed within 3 mo, and all three rats could hardly move because of the muscular atrophy of the hind legs. A most severely affected rat, WKA no. 54, and a moderately affected rat, WKA no. 57, were histologically examined. Provirus genomes in the central nervous system (CNS) tissues, as well as in the PBMC, and anti-HTLV-I antibodies in the serum and CSF, were also examined. The remaining one was found dead and an autopsy was not done. Macroscopically, the brains of both rats appeared normal. The spinal cord of WKA no. 54 showed slight atrophy, particularly in the thoracic region, while that of no. 57 appeared to be normal. On microscopic examination of no. 54, both myelin and axons in the lateral and anterior funiculi were extensively damaged in a symmetrical fashion, and were infiltrated with abundant foamy macrophages (Fig. 3, A-D). Marked vacuolar changes were observed, however, slight vacuolar changes were also observed in the age-matched untreated controls. The lesion developed preferentially in the lateral and anterior funiculi, and apparently to a lesser extent in the posterior funiculi. The most severe lesions were at levels of the thoracic cord, where they continued from the cervical to the lumbar cord and were localized in the outer portion of the funiculi. The schematic distribution of the affected lesion in the spinal cord is shown in Fig. 3 E. The inner portion of the funiculi and central gray matter appeared intact. Lymphocytic infiltration was virtually absent in the white and gray matter, or in the perivascular areas throughout the entire spinal cord. Fibers of the anterior and posterior roots of the severely affected cord also showed loss of myelin and axons with infiltration of foamy macrophages. There appeared to be no neuronal...
Table 4.  Tissue Distribution of HTLV-I Provirus Genome in F344 Rats

| Rat no. | Cerebrum | Cerebellum | Thymus | Heart | Lung | Liver | Kidney | Spleen | Submandibular gland | Lymph nodes | PBMC |
|---------|----------|------------|--------|-------|------|-------|--------|--------|----------------------|-------------|-------|
| 36      | -        | -          | +      | -     | +    | -     | +      | +      | +                    | -           | +     |
| 128     | -        | -          | +      | +     | +    | +     | -      | +      | +                    | -           | +     |
| 129     | +        | -          | +      | -     | -    | +     | +      | -      | -                    | -           | +     |
| 130     | +        | +          | +      | +     | -    | +     | +      | +      | +                    | +           | +     |
| 131     | +        | -          | +      | -     | -    | -     | +      | -      | +                    | -           | +     |

Discussion

Several observations presented in this paper lead to the conclusion that the inbred rat provides a useful animal model for HTLV-I infection in humans. In 1984, we reported that HTLV-I infected and immortalized rat T cells of the spleen, lymph nodes, and thymus, when cocultivated with BrdUrd-treated ATL cells (17). In the present study, by inoculating newly established rat T cell lines or a human T cell line, MT-2, we succeeded in establishing HTLV-I carriers in several inbred strains of newborn and adult rats, as determined by virus genome integration. We found no strain differences in the susceptibility to HTLV-I virus infection in neonates, and transmission efficiency exceeded 80%. Adult rats of several different strains are equally susceptible to HTLV-I infection. Newborn HTLV-I carriers, in general, show an unresponsiveness in humoral antibody response to HTLV-I throughout their life span. However, this unresponsiveness can be easily broken down, since a secondary challenge of MT-2 cells at 7 mo leads to the production of anti-HTLV-I antibodies with antibody titers of 1:256 to 1:512, as tested 1 mo later (our unpublished results). Adult HTLV-I carriers continuously produce humoral antibodies to HTLV-I similar to those in humans. According to antibody titers against HTLV-I, there are three groups of inbred rat strains: ACI, F344, and SDJ (high responders); WKA, BUF, and LEJ (intermediate responders); and LEW (low responders). In human carriers, there are high and low responders to HTLV-I; as it is possibly related to particular HLA haplotypes, the former is likely to become HAM/TSP (27). Therefore, strain difference in the humoral antibody response to HTLV-I in the rat provides useful information for future research.

Three of three newborn HTLV-I carriers of the WKA strain developed spastic paraparesis of the hind legs, at 16 mo. Clinical symptoms and neuropathological findings generally mimic those of HAM/TSP seen in humans (6–9, 28–30). The lesions are primarily confined to the lateral and anterior funiculi of the spinal cord. The most severe lesions are levels of the thoracic cord, and continue from the cervical to the lumbar cord. Both myelin and axons are extensively damaged, in a symmetrical fashion, and are infiltrated with massive foamy macrophages. Newborn and adult carriers of other strains...
Figure 3. Histopathological findings and schematic distribution of the affected lesion in the spinal cord of HAM rats. (A and B) Symmetrical distribution of the damage in the white matter is shown, as a whole view of the affected thoracic cord (no. 54, Th7) (×30). (C and D) Vascular degeneration, demyelination, and infiltration of foamy macrophages are evident in the enclosed area of A (×140). Distribution of affected lesions in spinal cords of nos. 54 and 57 are represented in the schematic figure (E). The solid area shows severe damage and the hatched area a lesser degree of damage. No marked change was found in the blank area, as compared with age-matched untreated control. (A and C) HE stain; (B and D) LFB stain.
have not developed neurological disease clinically and pathologically. Thus, HTLV-I carriers of the WKA strain are suitable models for the HAM/TSP in humans. The WKA/Hkm rats, formerly called Wistar-King-Aptekman rats, are agouti (A/A), coat color (B/B), and albino (c/c), and have been typed for the following polymorphic loci: Abp-1b, Aplr, Cst, Es-1, Es-2, Es-3, Es-4, Es-7, Es-8, Es-9, Es-10, Ert, Fk-1, Gst-1, Haa-1, Hbb, Mdl-1, Mpl, Pgd, RTI-1, Syp-1, Tdm-1. Since HAM/TSP occurs with increased frequency with certain HLA haplotypes (27), the genetic background of WKA, especially the RTI-1 haplotype of rat MHC, may be one of critical host factors determining disease susceptibility. Further investigations are necessary to clarify this point.

Retrovirus-induced neurological diseases have been recognized for many years in animals. Among them, retroviral spongiform polioencephalomyelopathy in wild mice (33-35) and visna in sheep (36-38) have been most extensively studied. From animal studies, there is evidence that retroviral infection can damage the CNS tissue via either immunological mechanisms or through a direct neurotropic effect of the retrovirus. The intense lymphocytic infiltration in the affected lesion is a characteristic pathologic feature of rat HAM. Whether the absence of lymphocytic infiltration in the affected lesion reflects the stage of the disease process, species difference in the host response between rats and humans, or nonimmunological demyelination in the pathogenesis of rat HAM remains to be determined. Third, HTLV-I provirus genome was clearly demonstrated by PCR in the spinal cord and cerebrum as well as PBMC of a ham rat no. 57. The precise localization of provirus genome, whether it localizes in the damaged spinal cord tissue, in the infiltrating macrophages, or in the blood-borne or hidden lymphocytes carrying HTLV-I, will remain to be determined. Neither integration of provirus genome, nor expression of mRNA and viral proteins of HTLV-I, has been confirmed in the diseased CNS tissues of HAM/TSP patients (29, 43). Therefore, there is no direct proof that the inflammation and tissue damage in the spinal cord of HAM/TSP patients is a consequence of viral infection in the CNS tissue. Determination of in situ localization of provirus genome, mRNA, and viral proteins in the affected spinal cord of the HAM rat would be of critical importance for investigating pathogenic roles of HTLV-I in the disease mechanism of HAM/TSP in humans.

In conclusion, this is the first description of a rat model for HAM/TSP in humans. This model is expected to contribute to a better understanding of mechanisms involved in the etiopathogenesis of HTLV-I-related immunological diseases in humans, particularly HAM/TSP.

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Address correspondence to Takashi Yoshiki, Department of Pathology, Hokkaido University School of Medicine, Kita-15 Nishi-7, Kita-ku, Sapporo 060, Japan.

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