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High Incidence of HAM/TSP-Like Symptoms in WKA Rats after Administration of Human T-Cell Leukemia Virus Type 1-Producing Cells

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We demonstrate a significantly high incidence of human T-cell leukemia virus type 1 (HTLV-1)-associated myelopathy (HAM)- or tropical spastic paraparesis (TSP)-like symptoms in WKA rats after injection with HTLV-1-producing MT-2 cells, while no symptoms were observed in F344 rats injected with MT-2 cells or in control WKA rats. Five of the eight (63%) WKA rats injected with MT-2 cells showed HAM/TSP-like paraparesis at 105 weeks of age, but none of seven MT-2-injected F344 rats or eight control WKA rats showed symptoms. This high incidence of HAM/TSP-like symptoms in WKA rats was statistically significant ($P < 0.05$). Six of the eight (75%) WKA rats injected with MT-2 cells showed HAM/TSP-like paraparesis at 108 weeks of age. HAM/TSP-like symptoms were also observed in one of the two WKA rats injected with HTLV-1-producing Ra-1 cells at 128 weeks of age. HTLV-1 provirus was detected in peripheral blood mononuclear cells in both WKA and F344 rats. The provirus was detected in the spinal cords of the HAM/TSP-like WKA rats that had severe neuropathological changes. WKA and F344 rats showed no significant difference in antibody response against HTLV-1 Gag antigen. However, the antibody response against the C-terminal half of gp46 HTLV-1 envelope protein was lower in WKA rats than in F344 rats. Pathological analysis of the HAM/TSP-like rats showed degeneration of the white matter of the spinal cord and peripheral nerves. These findings suggest that both the genetic background of the host and HTLV-1 infection are important in neuropathogenesis of HAM/TSP-like paraparesis in rats.

In 1985, Gessain et al. reported that a significant proportion of tropical spastic paraparesis (TSP) was pathogenetically related to human T-cell leukemia virus type 1 (HTLV-1) infection (13). In 1986, Osame et al. proposed a new clinical entity, HTLV-1-associated myelopathy (HAM), that showed spastic paraparesis (34). Presently TSP and HAM belong to the same clinical entity, called HAM/TSP (4, 38, 48). HAM/TSP is a slowly progressive disease that is shown by prominent pyramidal tract signs and mild sensory and sphincter disturbances (33). Less than 0.1% of HTLV-1 carriers develop HAM/TSP throughout their lifetimes (20). The involvement of genetic background in human HAM/TSP is suggested, but this has not been conclusive (47).

The amount of HTLV-1 provirus in peripheral blood mononuclear cells (PBMC) of HAM/TSP patients is reported to be greater than in HTLV-1 carriers (12, 25, 42). HTLV-1 provirus has been detected from the spinal cords of HAM/TSP patients by PCR (6, 24). However, it is not clear whether there are patients with HAM/TSP symptoms without HTLV-1 infection, since it has been reported that there are patients with TSP without HTLV-1 antibody (13, 48). Kira et al. could show no correlation between the amount of HTLV-1 provirus in PBMC of HAM/TSP patients and the severity of the disease (25).

Titers of antibody against HTLV-1 antigens in plasma and cerebrospinal fluid (CSF) in HAM/TSP patients were higher than those in HTLV-1 carriers (31, 33, 42). Levels of polyclonal immunoglobulins (Igs) (IgG and IgA) in serum were also elevated in HAM/TSP patients (19). The percentage of helper inducer T cells correlated with levels of spontaneous proliferation of peripheral blood lymphocytes and serum IgG in HAM/TSP patients (18). Cytotoxic T lymphocytes might induce demyelination of the spinal cord (22, 30). These findings suggest that host immune responses are involved in the neuropathogenesis of HAM/TSP. However, the mechanism of immune response in the neuropathogenesis of HAM/TSP is unclear.

Animal models of HAM/TSP would be useful for analysis of the pathogenesis and development of the measures for prevention and effective therapy of the disease. We have established an HTLV-1 carrier rat model by injection of an HTLV-1-producing human T-cell line (3, 43). In this study, to understand the roles of HTLV-1 in HAM/TSP, we have injected HTLV-1-producing T cells into WKA and F344 rats and studied the appearance of HTLV-1-associated symptoms in these rats. The involvement of both the genetic background and HTLV-1 in the pathogenesis of HAM/TSP is discussed.

MATERIALS AND METHODS

Animals and cell lines. WKA rats were purchased from Japan SLC, Inc., Shizuoka, Japan, and F344 rats were from

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Charles River, Kanagawa, Japan. HTLV-1-producing MT-2 cells, a human cell line (28), and Ra-1 cells, a rabbit cell line (29), were used. These cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. We injected 2.4×10^6 MT-2 cells twice intravenously at 3 and 4 weeks of age into both eight female WKA rats and eight female F344 rats. We also injected 4×10^7 Ra-1 cells intravenously into two 4-week-old female WKA rats.

PCR. The lysates of PBMC were prepared from the rats at various times after injection of HTLV-1-producing cells. DNA from the rat brain, spinal cord, lymph node, lung, liver, spleen, and kidney was prepared by sodium dodecyl sulfate (SDS)-proteinase K digestion, followed by phenol-chloroform–isoamyl alcohol extraction and ethanol precipitation. PCR was performed as described previously (43). Southern blot analysis of PCR products was performed according to the method of Albretsen et al. (2). We considered the HTLV-1 provirus to be present in the sample when at least one region (*gag*, *pX*, or long terminal repeat [LTR]) of HTLV-1 provirus was detected at least twice or when two regions of HTLV-1 provirus were detected in the same sample.

Antibody detection. The antibodies against HTLV-1 Gag proteins in the plasma and CSF were assayed with a commercially available particle agglutination kit (Serodia HTLV-1; Fujirebio, Tokyo, Japan) (16). The antibodies against HTLV-1 envelope proteins in the plasma were assayed by enzyme-linked immunosorbent assay (ELISA). N147 and C182 envelope proteins used as antigens were produced by a baculovirus expression system and were partially purified (32). N147 contains the C-terminal half of the external glycoprotein, gp46, and C182 contains almost the entire region of the transmembrane glycoprotein, p20E (32, 44). Western blot (immunoblot) analysis was performed by a standard method to detect antibodies against HTLV-1 Gag proteins (8, 46). MT-2 cell lysates were separated by SDS-polyacrylamide gel electrophoresis and transferred to a nylon membrane (Immobilon; Millipore). The plasma of rats, or that of an adult T-cell leukemia patient, was used as the primary antibody. Biotinylated anti-rat IgG(H+L) (Bio-Rad) or biotinylated anti-human IgG(H+L) (Bio-Rad) was used as the secondary antibody.

Histopathological analysis. The rats were anesthetized with ethyl ether and perfused with 2% paraformaldehyde (pH 7.2) at autopsy. The brain and spinal cord were fixed in 4% cacodylate-buffered paraformaldehyde (pH 7.2) or periodate–lysine–4% paraformaldehyde (pH 7.4). Other organs were fixed in 4% cacodylate-buffered paraformaldehyde (pH 7.2). Three-micrometer-thick paraffin sections were stained with hematoxylin and eosin. Sections from nervous tissues were further stained by the Klüver-Barrera method or LFB-PAS and by the modified Bielschowsky or Bodian method (27). The paraffin sections were also immunostained by using the anti-pan-T staining method (R1-3B3; Seikagaku Co.), antimacrophage antibodies (ED-1; Selotec), and anti-glial fibrillary acidic protein antibodies (Z344; DAKO). The spinal roots and sciatic nerves of some rats were fixed in 2.5% glutaraldehyde, postfixed with 1% osmium tetroxide, and embedded in epoxy resin. One-micrometer-thick sections were stained with toluidine blue. After fixation with osmium, other parts of the peripheral nerves were examined by a teasing method.

Statistics. We analyzed the frequency of HAM/TSP-like paraparesis and that of HTLV-1 provirus in PBMC by Fisher's exact test (39).

TABLE 1. Rats with spastic paraparesis after injection with HTLV-1-producing T-cell lines

Strain	Cell line	No. of cells	No. of rats	No. of paraparetic rats	Rat no.	Age at onset (wks)	Age killed (wks)
WKA	MT-2	4.8×10^6	8	6	TW90		119[a]
					TW91	82	103
					TW92	83	85
					TW93	82	84
					TW94		77
					TW95	105	107[a]
					TW96	105	107[a]
					TW97	118	122
	Ra-1	4.0×10^7	2	1	GW70		119
					GW71	128	132
	None	NA[b]	10	0	TW11		113[a]
					TW12		96[a]
					TW13		>120[c]
					TW14		>120[c]
					TW15		>120[c]
					TW16		101[a]
					TW17		>120[c]
					TW18		118
					TW19		>120[c]
					TW20		>120[c]
F344	MT-2	4.8×10^6	8	0	TF80		105[a]
					TF81		49[a]
					TF84		105[a]
					TF85		114
					TF86		107
					TF87		114
					TF88		114
					TF89		114

[a] Rat died accidentally.
[b] NA, not applicable.
[c] Rat is surviving.

RESULTS

High incidence of HAM/TSP-like symptoms in WKA rats. WKA and F344 rats, except TF81, injected with HTLV-1-producing cells were monitored for 2 years (Table 1). At 105 weeks of age, five HTLV-1 carrier WKA rats (63%) injected with MT-2 cells showed HAM/TSP-like paraparesis, while none of the eight F344 rats injected with MT-2 cells and none of the eight control female WKA rats not injected with MT-2 cells showed the paraparesis. The incidence of the HAM/TSP-like paraparesis was significantly higher in HTLV-1 carrier WKA rats than in HTLV-1 carrier F344 rats ($P < 0.05$; Fisher's exact test) and control WKA rats ($P < 0.05$) at 105 weeks of age (Table 1). From 82 to 118 weeks of age, six (TW91, -92, -93, -95, -96, and -97) of the eight female WKA rats (TW90 to TW97) showed spastic paraparesis and muscle atrophy in their posterior limbs and hips, with fine and coarse tremulous movement. Their anterior limbs appeared slightly paretic. Paraparesis and gait disturbance were slowly progressive. Five of the eight WKA rats injected with MT-2 cells were autopsied; the other three rats, TW90, -95, and -96, died accidentally (Table 1). TW94 did not show spastic paraparesis but was sacrificed because of a subcutaneous tumor (40 by 35 mm) at 77 weeks of age. One of the two female WKA rats injected with Ra-1 cells, GW71, showed paraparesis and gait disturbance at 120 weeks of age and was sacrificed at 132 weeks of age (26). A female F344 rat injected with MT-2 cells died accidentally at

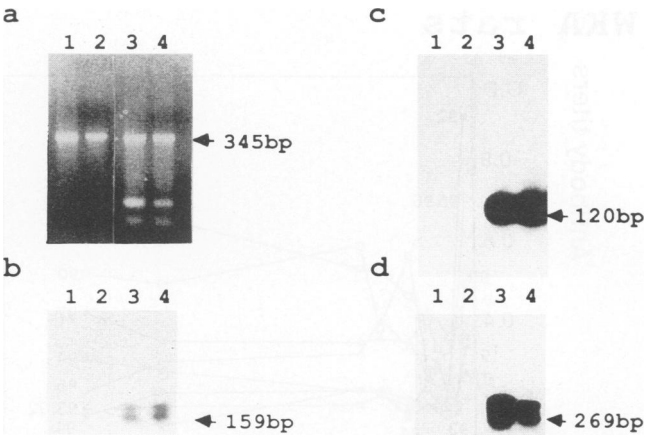

FIG. 1. Detection of HTLV-1 provirus sequences in PBMC from the HAM/TSP-like rat, TW92, at the 81st week after injection with MT-2 cells. (a) Ethidium bromide staining of agarose gel. Rat c-*myc* sequences are shown. Panels b, c, and d are autoradiographs indicating amplification of *pX*, *gag*, and LTR sequences, respectively. Lanes 1 and 2, normal PBMC lysate; lanes 3 and 4, PBMC lysate from TW92.

49 weeks of age. The other seven female F344 rats did not show any neurological abnormalities during the observation period until 114 weeks of age (Table 1).

Detection of HTLV-1 provirus. We analyzed PBMC of the rats injected with MT-2 cells for the presence of HTLV-1 provirus until the 81st week after MT-2 cell injection. HTLV-1 provirus was detected from PBMC of all WKA rats at least twice after HTLV-1 injection (Fig. 1). No HTLV-1 provirus was detected from PBMC of negative-control rats which had not received injection of HTLV-1-producing cells. We found HTLV-1 provirus in eight of eight WKA rats and five of eight F344 rats injected with MT-2 during the course of the observation period. The frequency of detection of the provirus in PBMC of WKA and F344 rats was not statistically significant by Fisher's exact test ($P = 0.1$). Among HAM/TSP-like rats, HTLV-1 provirus was detected from the brain, the spinal cord, and the lymph nodes of GW71; the lymph nodes of TW91; and the spinal cord of TW97.

Serological analysis. Antibodies against HTLV-1 Gag proteins were detected by the particle agglutination method in plasma of all rats after injection with HTLV-1-producing cells (Fig. 2). Titers of the antibody increased to reach a peak at about the 10th week after MT-2 cell injection in both WKA and F344 rats. Titers of antibody against Gag in the plasma of the five HAM/TSP-like rats ranged from 16- to 64-fold, and those in CSF were 32-fold in TW92 and TW93 and less than 16-fold in GW71 and TW97 at autopsy. By Western blot analysis, antibodies against HTLV-1 Gag proteins p19, p24, p28, and p51 were found in the plasma of WKA and F344 rats injected with MT-2 cells at the 12th week after MT-2 cell injection and those against p24 and p28 were detected at autopsy (data not shown).

All the rats injected with MT-2 cells had antibodies against HTLV-1 envelope proteins, N147 (Fig. 3) and C182 (Fig. 4). Titers of the antibodies against these envelope proteins decreased rapidly by the second or the third week after MT-2 cell injection but gradually increased thereafter. Titers in F344 rats after the 10th week following MT-2 cell injection were higher than those in WKA rats (Fig. 3). There was no apparent difference in the titers of antibody against C182 between WKA and F344 rats (Fig. 4).

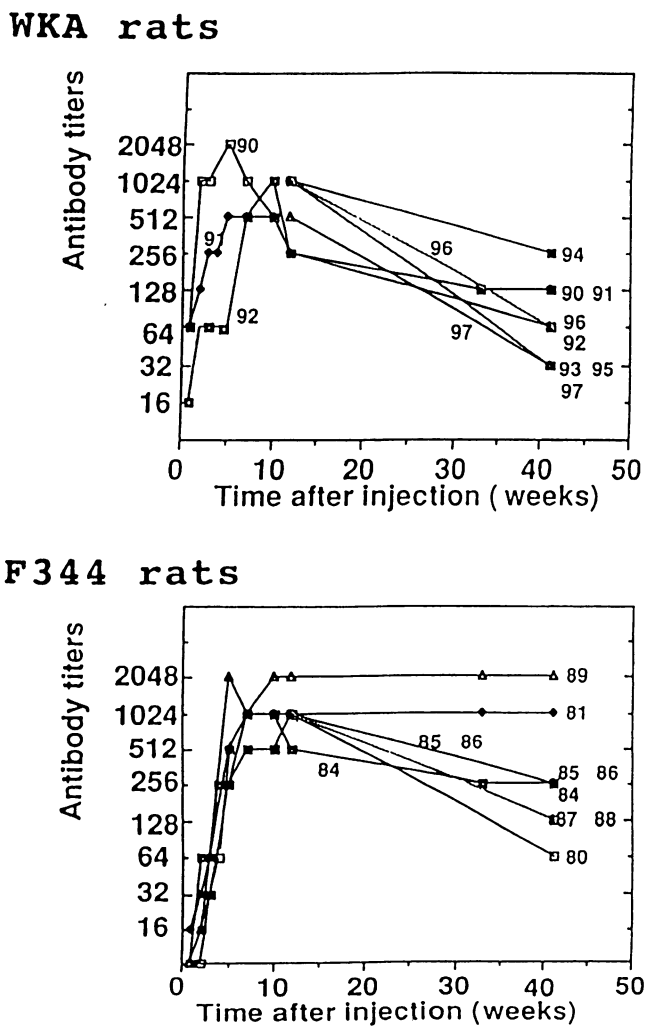

FIG. 2. Antibody response against HTLV-1 Gag in WKA and F344 rats injected with MT-2 cells. Numbers 90 through 97 and 80 through 89 are TW and TF rat numbers, respectively.

Pathological findings for HAM/TSP-like rats. The spinal cords of the four HAM/TSP-like rats (TW91, TW92, TW93, and TW97) showed symmetrical white matter degeneration with vacuolation and gliosis which were prominent in the internal posterior funiculi (Goll funiculi) and marginal regions of the anterior and lateral funiculi, as was observed for GW71 (26). The grey matter was almost normal, and the neurons were well preserved. Perivascular or subarachnoidal cell infiltration was absent. The degree of degeneration in the spinal cord was severe in the cervical and thoracic cords but varied among the rats. We confirmed infiltration of macrophages by staining with antimacrophage antibodies, ED-1, and increase of reactive astroglia in the degenerated white matter by staining with anti-glial fibrillary acidic protein antibodies. We did not detect lymphocytes in the spinal cords with antibody against pan-T cells, R1-3B3. No neuropathological findings in the brain were made, except pituitary adenocarcinomas in TW92 and TW93 (7 by 8 mm in TW92 and 5 by 7 mm in TW93). Peripheral nerves—anterior and posterior nerve roots, the cauda equina, and the sciatic nerve—in the HAM/TSP-like rats showed vacuolation and loss of large and small myelinated

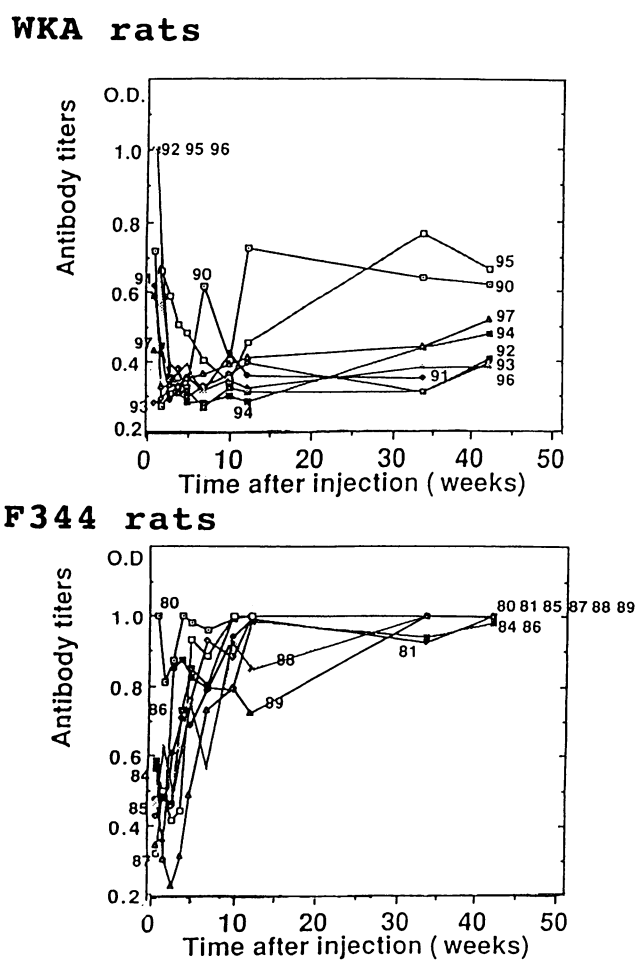

FIG. 3. Antibody response against HTLV-1 envelope protein N147 in WKA and F344 rats injected with MT-2 cells, as determined by ELISA. O.D., optical density at 492 nm. Numbers 90 through 97 and 80 through 89 are TW and TF rat numbers, respectively.

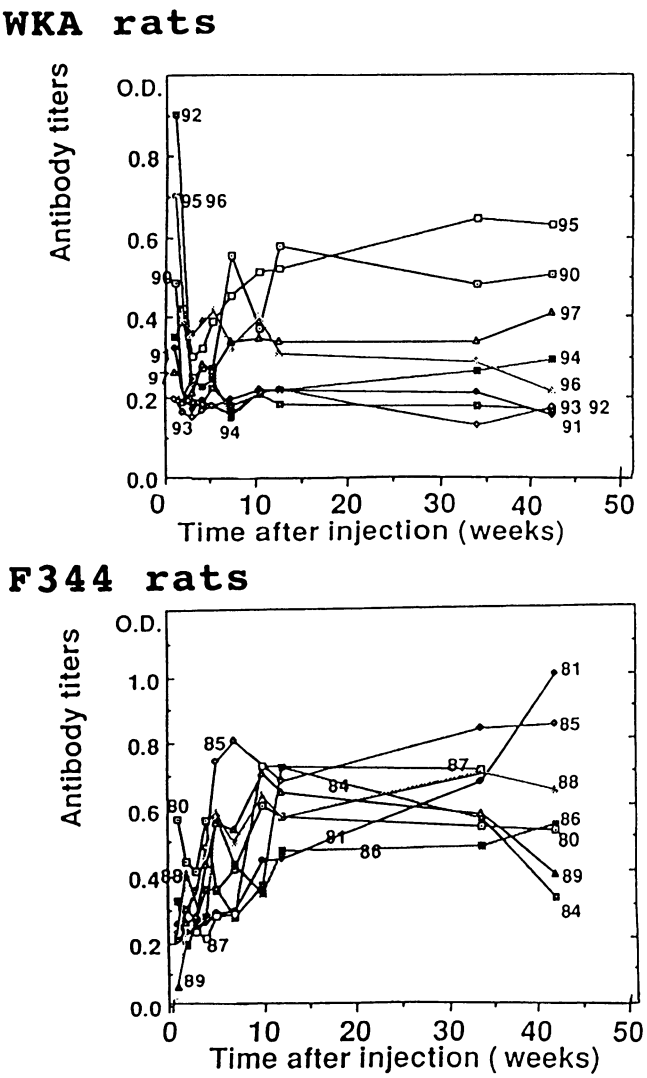

FIG. 4. Antibody response against HTLV-1 envelope protein C182 in WKA and F344 rats injected with MT-2 cells, as determined by ELISA. O.D., optical density at 492 nm. Numbers 90 through 97 and 80 through 89 are TW and TF rat numbers, respectively.

fibers. In sciatic nerves of TW91 and TW97, we found infiltration of foamy macrophages and multinucleated giant cells in the areas exhibiting severe loss of myelinated fibers. These pathological changes in the peripheral nerves will be reported elsewhere. Scattered angular fibers and grouped atrophy were found in the psoas muscle of the five HAM/TSP-like rats.

Stomach ulcers were found in TW91 and TW92. Abscesses were found in the space between the bladder and uterus in TW92 and TW93.

DISCUSSION

This study showed that the incidence of paraparesis was significantly higher in WKA rats injected with MT-2 cells than in F344 rats injected with MT-2 cells or in uninjected control WKA rats. This result indicates that the genetic background of the host and HTLV-1 infection are important for the appearance of HAM/TSP-like symptoms in rats.

Several characteristics of HAM/TSP-like rats are similar to those of HAM/TSP patients. First, HTLV-1 provirus was detected in PBMC and the spinal cord, and antibody against HTLV-1 proteins was found in plasma and CSF. Although the continuing presence of MT-2 cells was not completely excluded, the long-lasting antibody against HTLV-1, which lasted for more than 1 to ~1.6 years, suggests the productive infection of HTLV-1. This is consistent with our previous observations (43). Second, the genetic background seems to be related to the occurrence of the disease. The major histocompatibility complex in rats, RT-1, is of the *k* haplotype in WKA rats, while that of F344 rats is of the *lv1* haplotype (14). Usuku et al. reported HLA haplotype-linked high-level immune responsiveness against HTLV-1 in HAM/TSP patients (47). The incidence of HAM/TSP would be much higher if we selected HTLV-1 carriers with a specific genetic background. Third, the main symptoms are paraparesis. The neuropathological changes are pronounced in cervical and thoracic cords, and they are mainly demyelination with vacuolation and gliosis. The peripheral nerve lesions and muscle involvements, which were frequently found in the HAM/TSP-like rats, were recently reported to be not uncommon in HAM/TSP patients (5, 40).

Recently, Ishiguro et al. reported HAM/TSP-like myelopa-

thy rats without anti-HTLV-1 antibody in the blood (17). They described the presence of HTLV-1 provirus in PBMC, the cerebrum, and the spinal cord of one of the three paraparetic WKA rats 16 months after MT-2 cell injection. The other organs were mostly negative. We also found the presence of HTLV-1 provirus in PBMC of all MT-2-injected WKA rats and in the brains, spinal cords, and lymph nodes of three of the seven paraparetic WKA rats. These findings agree well with the report by Ishiguro et al. (17). Moreover, we observed that the *pX* region of the provirus in the spinal cord was found in the two most severely affected rats. HTLV-1 provirus was detected from the spinal cords of HAM/TSP patients by PCR (6, 24). However, the target cell in which HTLV-1 provirus integrated remains to be identified for the spinal cords of both the patients and the HAM/TSP-like rats. By in situ PCR, HTLV-1-infected cells may be identified in organs, as with human immunodeficiency virus (HIV) (10, 36).

Immune responses are involved in HAM/TSP (18, 21). Titers of antibody against HTLV-1 Gag in plasma and CSF in HAM/TSP patients are higher than those in HTLV-1 carriers (31, 33, 42). However, the titers of antibody against Gag did not differ between WKA and F344 rats. On the other hand, titers of antibody against N147 of the Env product were lower in WKA rats than in F344 rats. N147 contains the C-terminal 147-amino-acid half of the gp46 external envelope glycoprotein of HTLV-1 (44). The putative neutralizing epitopes of gp46 (35, 45) are included in N147. Therefore, a low humoral immunity response to the gp46 envelope protein may be involved in the pathogenesis of HAM/TSP-like symptoms in WKA rats. Ishiguro et al. have reported HAM/TSP-like rats without antibody against HTLV-1 Gag in plasma (17). However, the antibodies against the HTLV-1 gene products other than Gag, or the antibodies against self antigens which are induced by HTLV-1 antigens, might have some role in pathogenesis of HAM/TSP. It should be noted that the transgenic mice expressing the *env* gene of Cas-Br-E murine retrovirus in the spinal cord showed spongiform degeneration of the spinal cord, causing hind-limb paresis (23).

Immunostaining and histochemical staining revealed the infiltration of macrophages and increase of astroglia in the degenerative nervous tissues of the HAM/TSP-like rats, as in HAM/TSP patients (20). The infiltration of macrophages and the appearance of multinucleated giant cells in the spinal cord observed in HAM/TSP-like rats are also observed in patients with HIV encephalopathy (7). HTLV-1 Tax protein induces release of tumor necrosis factor alpha (TNFα) from macrophages (1), and TNFα causes white matter degeneration by injury to oligodendrocytes (41). Astrocytes might present antigens such as myelin basic protein (11) or release factors cytotoxic for oligodendrocytes (37). Lymphocytes were found in the spinal cords of HAM/TSP patients at the early stage but were not found at the late stage (20). Lymphocytes might be found at an earlier stage in our HAM/TSP-like rats.

Most of the HAM/TSP patients were given steroid therapy, and some of the patients have another disease, such as cancer (20). The drugs and treatments for HAM/TSP and those for coexistent diseases could modify the virological and neuropathological findings for HAM/TSP patients (9, 15). Our HAM/TSP-like rats should be a useful small-animal model to analyze the roles of HTLV-1 and various other factors involved in the wide diversity of neuropathological changes associated with HAM/TSP. Involvement of genetic background in the neuropathogenesis of HAM/TSP should be clarified by experiments with congenic rats. It would be interesting to know whether other species of the major histocompatibility complex (RT-1) *k* genotype display a susceptibility to HAM/TSP-like paraparesis similar to that of the WKA rats. Furthermore, this rat model should be helpful to develop effective measures for prevention and treatment of HAM/TSP.

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