Oral Administration of Human T-Cell Leukemia Virus Type 1 Induces Immune Unresponsiveness with Persistent Infection in Adult Rats

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The major route of human T-cell leukemia virus type 1 (HTLV-1) infection is mother-to-child transmission caused by breast-feeding. We investigated the host immune responses to orally established persistent HTLV-1 infection in adult rats. HTLV-1-producing MT-2 cells were inoculated into immunocompetent adult rats either orally, intravenously, or intraperitoneally. HTLV-1 proviruses were detected in the peripheral blood and several organs for at least 12 weeks. Transmission of HTLV-1 to these animals was confirmed by analysis of HTLV-1 flanking regions. Despite persistent HTLV-1 presence, none of the orally inoculated rats produced detectable levels of anti-HTLV-1 antibodies, whereas all intravenously or intraperitoneally inoculated rats showed significant anti-HTLV-1 antibody responses. T-cell proliferative responses against HTLV-1 were also absent in orally inoculated rats. Our findings suggest that gastrointestinal exposure of adult rats to HTLV-1-infected cells induces persistent HTLV-1 infection in the absence of both humoral and cellular immune responses against HTLV-1. This immune unresponsiveness at primary infection may subsequently affect the host defense ability against HTLV-1.

Human T-cell leukemia virus type 1 (HTLV-1) is a human retrovirus associated with T-cell malignancies (5, 26). Most HTLV-1-infected individuals remain asymptomatic, and less than 5% develop adult T-cell leukemia, HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), or other HTLV-1-associated diseases (11, 32). A number of studies have shown that the variable clinical outcome of HTLV-1 infection cannot be explained by different genetic forms of HTLV-1 strains (2, 16, 17). Instead, the pathogenesis of HTLV-1 is more likely to be influenced by other factors, such as oncogenic mutations and host factors.

One of the host factors that may determine the development of diseases is the level of the immune response to HTLV-1 in individual subjects. For example, the activity of HTLV-1-specific cytotoxic T lymphocytes in peripheral blood is low in adult T-cell leukemia patients but high in HAM/TSP patients (8–10). In the presence of a weak cytotoxic-T-lymphocyte response, HTLV-1 can easily replicate and the infected cells may have better chances to multiply and acquire an autonomously proliferative character. Admittedly, the exact mechanisms resulting in different immune responses to HTLV-1 are still unclear. Involvement of the genetic background and immunological tolerance in HTLV-1 carriers have been suggested (36). A number of vertically HTLV-1-infected individuals lack antibody responses to HTLV-1 during infancy (27), supporting the notion of tolerance for HTLV-1 infection.

The transmission routes for HTLV-1 include mother-to-child transmission, sexual contact, and parenteral transmission through blood transfusion or intravenous drug use (4, 13, 25, 31). Among these, mother-to-child transmission is the major natural transmission pathway in Japan (4, 13, 23). HTLV-1 is detected in breast milk from carrier mothers and sometimes in the cord blood (28). The infants of these mothers are reported to be fed about 10^8 HTLV-1-infected cells before weaning (14, 23). In contrast, bottle feeding prevents most infants from acquiring HTLV-1 infection (1), indicating that postnatal infection by breast-feeding is the major form of mother-to-child transmission of HTLV-1, although prenatal infection also occurs, but at a low frequency.

Oral administration of protein antigens is known to induce peripheral tolerance for the fed antigens (3, 39). Since HTLV-1 is transmitted to infants mainly via breast milk, gastrointestinal exposure to HTLV-1 could be one reason for immunological tolerance for HTLV-1. A few studies showed that oral administration of HTLV-1-producing cells transmitted HTLV-1 to common marmosets and rabbits (15, 35, 41). However, no studies have fully characterized the immunological responses in orally HTLV-1-infected animals.

In the present study, we investigated the immune responses to HTLV-1 in adult rats orally inoculated with HTLV-1-producing cells and found a persistent HTLV-1 infection in the absence of both humoral and cellular immune responses. Our results indicate that the immune unresponsiveness in oral HTLV-1 infection may be one of the determinants affecting the host defense system against HTLV-1.

MATERIALS AND METHODS

Animals and inoculation of HTLV-1. Inbred female F344/N Jcl-mui/ + rats were purchased from Clea Japan, Inc. (Tokyo, Japan). A human HTLV-1-infected T-cell line, MT-2, was used as the viral source.

For oral inoculation, 5 × 10^7 MT-2 cells were administrated to four rats through a feeding tube. Another group of three animals was intraperitoneally injected with 10^7 MT-2 cells once, while six other animals were intravenously injected with 10^7 MT-2 cells once.
injected with the same number of MT-2 cells twice at a 1-week interval. All rats were inoculated at 4 weeks of age. Samples of peripheral blood cells were collected from each rat every other week after inoculation, and levels of HTLV-1 provirus in blood cells and antibodies to HTLV-1 in sera were measured. The animals were sacrificed at 3 months after inoculation, and the presence of HTLV-1 provirus in various tissues was examined. The experimental protocol was approved by the Animal Care Committee of Tokyo Medical and Dental University.

Detection of HTLV-1 provirus. HTLV-1 provirus in peripheral blood and tissues was detected by the nested PCR method. For this purpose, 3 μl of each whole blood sample was lysed in a Gene trap solution (single-tube PCR kit; Takara, Kyoto, Japan), precipitated, and used as a template for PCR amplification. DNA samples from organ tissues were prepared by sodium dodecyl sulfate and proteinase K digestion, purification with phenol-chloroform, and ethanol precipitation. A 0.5-μg quantity of each DNA sample was used as a PCR template.

PCR amplification with HTLV-1 pX-specific primers pX1 (5'-CCCACTTCC CAGGGTTTGGCACAGGTCCT3') and pX4 (5'-CCGATACCAAGCTCTCA CGTGGTTTGGAGACTGT3') was performed with 30 cycles of denaturation (95°C, 1 min), annealing (60°C, 1 min), and polymerization (72°C, 1 min). A portion of the PCR product was reamplified with inner primers pX2 (5'-GAGCCGAT AAGCGGCCCTGAGTGCC3') and pX3 (5'-GGCGGAGGAGG GCGTGAAGAAGAAA3'). An HTLV-1 gag-specific outer primer set, gag-Os (5'-GCAGACCATCCGGCTTGCGG3') and gag-OR (5'-TGTCATT CTCCGCCAATAACC3'), and an inner primer set, gag (5'-AGCACTTTGACCCATCTGGAACCTCAAACTCCAGCTCTCCAGTTCTCTT3') and gag' (5'-GTGGTCTGTTGATTTGGC3'), were also used for nested PCR. The PCR products were analyzed by 3% agarose gel electrophoresis.

Analysis of HTLV-1 flanking regions. HTLV-1 flanking regions of MT-2 cells were obtained by the inverse PCR method as described by Takemoto et al. (33). Briefly, Sun1AI-digested cellular DNA of MT-2 cells was self-ligated and ammended with HTLV-1 long terminal repeat-specific primers. The fragments were cloned, and the sequence of one of the clones was determined by the dideoxy method with an Applied Biosystems DNA sequence kit. Primers MT2-1 (5'-TC CTCCAGTGACGGCGCGTG3') and MT2-2 (5'-GTTAATCCTCGGTCCAGC GG3') were prepared based on the obtained HTLV-1 flanking sequence of MT-2 cells. The HTLV-1 long terminal repeat-specific primers were used US-4 (5'-CCAGGACAGCGCCATTCTT3') and US-5 (5'-CTCAAGGAGAAA TTTAGTAC3'). Nested PCR amplifications were performed with outer primer set MT2-1 and US-4 and inner primer set MT2-2 and US-5 for 30 cycles with each set under conditions similar to those described above.

Detection of antibodies against HTLV-1 antigens. The titers of serum antibodies against HTLV-1 antigens were determined with a particle agglutination method (PHA) (Difco Laboratories, Detroit, Mich.) was used at a final 1% concentration as a positive control. Responder cells (105 per well) were cultured in a 96-well round-bottom plate in the presence or absence of equal numbers of stimulator cells for 96 h in triplicate. Finally, [3H]thymidine (37 kBq/well) was added during the last 12 h, the cells were harvested on a glass filter, and thymidine incorporation into the cells was measured. The results were expressed as the mean counts per minute of triplicate cultures ± the standard deviation. Thymidine uptake into formalin-treated cells was less than 30 cpm.

RESULTS

Oral inoculation of HTLV-1 does not produce antibody responses. To compare the immune responses to HTLV-1 in animals infected through various routes, we inoculated HTLV-1-producing MT-2 cells orally, intravenously, or intraperitoneally into 4-week-old immunocompetent F344/N Jcl-mu/+ rats. The serial changes in antibody titers to HTLV-1 in these animals are shown in Fig. 1. All intravenously or intraperitoneally inoculated rats produced anti-HTLV-1 antibodies as early as 2 to 4 weeks after inoculation, and the antibody titers gradually increased during the observation period of 12 weeks. In contrast, none of the four orally inoculated rats showed such an anti-HTLV-1 antibody response. Although some of these animals were monitored for up to 16 weeks after inoculation, the titers remained below the detection levels during that period.

Presence of HTLV-1 provirus in inoculated rats. Although several inbred strains of adult rats are known to show persistent infection with HTLV-1 after intravenous inoculation with HTLV-1-producing cells (6, 30), little is known about orally inoculated rats. Accordingly, we used the PCR method to assess whether the rats inoculated with MT-2 cells were infected with HTLV-1. DNA extracted from 3 μl of each whole peripheral blood sample was used as a template for PCR amplification. At 6 to 8 weeks after inoculation, HTLV-1 provirus was detected in peripheral blood samples from four of the four orally, three of the three intraperitoneally, and five of the six intravenously inoculated rats (Table 1). After that, the presence of the provirus was confirmed in all animals during the period of observation or at autopsy. These findings indicate that HTLV-1 disseminates systemically in orally inoculated rats as well as intravenously or intraperitoneally inoculated ones. A similar seronegative HTLV-1 carrier state was induced in WKA/HKm rats by oral administration of MT-2 cells (data not shown).

Tissue distribution of HTLV-1. The systemic dissemination of HTLV-1 in orally inoculated rats was also demonstrated by PCR analysis of DNA samples from various organs with primers specific for the pX and gag regions. HTLV-1 proviruses were present in tissues from two orally inoculated rats (E3 and E4) sacrificed at 3 months after inoculation (Fig. 2). HTLV-1 provirus was detected in various organs, including the submandibular gland, thymus, lungs, liver, spleen, lymph nodes, Peytersons, and the thymus. The dissemination of HTLV-1 was also confirmed in the spleen and lymph nodes of orally inoculated rats. The presence of HTLV-1 provirus in oral tissues was detected by the nested PCR method. For this purpose, 3 μl of each whole blood sample was lysed in a Gene trap solution (single-tube PCR kit; Takara, Kyoto, Japan), precipitated, and used as a template for PCR amplification. At 6 to 8 weeks after inoculation, HTLV-1 provirus was detected in peripheral blood samples from four of the four orally, three of the three intraperitoneally, and five of the six intravenously inoculated rats (Table 1). After that, the presence of the provirus was confirmed in all animals during the period of observation or at autopsy. These findings indicate that HTLV-1 disseminates systemically in orally inoculated rats as well as intravenously or intraperitoneally inoculated ones. A similar seronegative HTLV-1 carrier state was induced in WKA/HKm rats by oral administration of MT-2 cells (data not shown).

<table>
<thead>
<tr>
<th>Route</th>
<th>No. of rats</th>
<th>Presence* of HTLV-1 provirus at wk after inoculation:</th>
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* The presence of HTLV-1 provirus in 3 μl of peripheral blood was detected by a pX-specific PCR method and is expressed as number of HTLV-1-positive rats/number of tested rats. NP, not performed.

FIG. 1. Oral inoculation of HTLV-1 failed to induce antibody responses in rats. HTLV-1-producing MT-2 cells were inoculated orally (■); intravenously (▲); and intraperitoneally (○) into four, six, and three F344/N Jcl-mu/+ rats, respectively. The anti-HTLV-1 antibody titers in the sera of these animals were determined by the particle agglutination method. Data are the averages of the titers ± standard deviations for each group. 2^o, log₂.

TABLE 1. Detection of HTLV-1 provirus in peripheral blood from HTLV-1-inoculated rats

<table>
<thead>
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<th>No. of rats</th>
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* The presence of HTLV-1 provirus in 3 μl of peripheral blood was detected by a pX-specific PCR method and is expressed as number of HTLV-1-positive rats/number of tested rats. NP, not performed.
er’s patches, and peripheral blood mononuclear cells, in rat E3 (Fig. 2A). Although a small amount of HTLV-1 was present in animal E4 relative to the other animal, the provirus was detected in the lymphoid tissues and submandibular gland (Fig. 2A). However, HTLV-1 provirus was below the detection level in the brain and kidneys of both rats.

**Lack of long-term MT-2 survival in orally inoculated rats.** To exclude the possible long-term survival of MT-2 cells in vivo in rats orally inoculated with HTLV-1, we analyzed the HTLV-1 flanking regions in infected cells of these animals. For this purpose, we cloned HTLV-1 flanking regions of MT-2 cells by using an inverse PCR method. Based on the nucleotide sequences of these regions, we prepared primers to specifically amplify the HTLV-1 flanking regions of MT-2 cells.

As shown in Fig. 3, by use of the nested PCR method, DNA fragments specific for pX and HTLV-1 flanking regions of MT-2 cells were amplified with a DNA template of MT-2 cells. In contrast, no fragment specific for the HTLV-1 flanking regions of MT-2 cells was amplified with a DNA template of the submandibular gland from an orally inoculated rat, whereas pX-specific fragments could be amplified with this template as well as the MT-2 template. These results suggested that HTLV-1-infected cells in orally inoculated rats differed from

**Lack of T-cell proliferative responses against HTLV-1 after oral inoculation.** In the next step, we examined T-cell proliferative responses against HTLV-1 antigens in HTLV-1-inoculated animals. A syngeneic rat HTLV-1-infected cell line, FPM-1, was used for HTLV-1 antigen-presenting cells. T-cell-enriched spleen cells from orally, intravenously, and intraperitoneally inoculated or naive rats were collected and incubated in the presence or absence of formalin-treated FPM-1 cells, and thymidine incorporation in these cells was measured.

Figure 4 shows the results for four representative animals. Spleen cells from orally inoculated rat E2 hardly proliferated in response to HTLV-1 antigens but proliferated with PHA stimulation. A similar pattern of T-cell proliferation was observed for another two orally inoculated rats and was indistinguishable from that of naive animals. In contrast, there was a significant proliferative response of T cells to HTLV-1 in in-

![FIG. 2. Detection of HTLV-1 provirus in orally inoculated rats by nested PCR amplifications with HTLV-1 pX (A)- and gag (B)-specific primers. (A) Tissue distribution of HTLV-1 in two orally inoculated rats, E3 (top) and E4 (bottom), at 3 months after inoculation. The presence of HTLV-1 provirus in 0.5 μg of DNA extracted from each indicated organ tissue was assessed by the nested PCR method. Rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used as an internal control. (B) DNAs (0.5 μg) from lymph nodes and peripheral blood mononuclear cells of orally inoculated rat E3 (lanes 1 and 2, respectively) and rat E4 (lanes 3 and 4, respectively) were used as templates for the nested PCR method.

![FIG. 3. Lack of MT-2 cell-specific HTLV-1 flanking regions in orally inoculated rats. DNA (0.5 ng) of MT-2 cells (lane 1) and DNAs (0.5 μg) of submandibular glands of orally inoculated rat E3 (lanes 2 and 3) and rat E4 (lane 3) were used as templates for nested PCR amplifications with primers amplifying the HTLV-1 flanking region of MT-2 cells (top) and primers amplifying the HTLV-1 pX region (bottom).

![FIG. 4. T-cell proliferative responses against HTLV-1 antigens in inoculated rats. T-cell-enriched spleen cells from NC (naive control), E2 (orally inoculated), D6 (intravenously inoculated), and G4 (intraperitoneally inoculated) rats were incubated in the presence (solid bars) or absence (open bars) of formalin-treated FPM-1 cells, and thymidine incorporation into cells was measured. PHA was used as a positive control (hatched bars). Data represent the mean counts per minute of triplicate cultures ± standard deviations.](http://jvi.asm.org/)

MT-2 cells but originated from host cells transmitted with HTLV-1 in vivo. Thus, oral administration of MT-2 cells transmitted HTLV-1 to the rats and induced a persistent HTLV-1 infection without an antibody response.
travenously or intraperitoneally inoculated rats. The pattern of T-cell proliferation in these animals was divided into two types, irrespective of the route of inoculation. The first pattern was observed for four animals and is represented by the results for rat D6 (Fig. 4), showing the proliferation of spleen T cells even in the absence of stimulation; this proliferation was hardly influenced by HTLV-1 stimulation. The second pattern was observed for four animals and is represented by the results for rat G4 (Fig. 4), showing a strong proliferative response of spleen T cells to HTLV-1 antigens. The response was confirmed to be specific for HTLV-1 by demonstrating that it was not induced by formalin-treated syngeneic rat simian virus 40-transformed cells (data not shown). Thus, orally HTLV-1-inoculated rats showed cellular as well as humoral immune unresponsiveness to HTLV-1 antigens.

**DISCUSSION**

The major finding of the present study was that oral administration of HTLV-1-infected cells induced both persistent HTLV-1 infection and immune unresponsiveness to HTLV-1. Orally inoculated rats completely lacked both antibody and T-cell responses to HTLV-1 antigens. In contrast, these responses were detected in intravenously or intraperitoneally inoculated rats. HTLV-1 provirus was detected in peripheral blood from orally inoculated rats as frequently as in that from intravenously or intraperitoneally inoculated rats, indicating that the quality of the anti-HTLV-1 immune responses but not viral persistence was affected by the route of HTLV-1 transmission.

Since the major cause of HTLV-1 infection is breast-feeding of infants by carrier mothers, it is possible that oral tolerance may occur in humans. It should be noted, however, that there are certain differences between our experimental design and natural milk-borne infection of children. For example, milk-borne HTLV-1 transmission in humans occurs after multiple low doses provided over a period of a few months, whereas we inoculated HTLV-1-infected cells into rats orally in a single dose. In addition, we used adult rats, while HTLV-1 infection occurs in neonates in humans. Therefore, neonatal tolerance and maternal antibodies may modify host immunity in humans.

Various animal models of HTLV-1 infection have been described. A seronegative HTLV-1 carrier state is produced in rabbits and rats by intraperitoneal inoculation with HTLV-1-producing cells during the neonatal period (6, 29). The development of the seronegative carrier state in these animals is thought to be due to immaturity of the immune system. Other investigators demonstrated that HTLV-1 could be orally transmitted to common marmosets and rabbits (15, 35, 41). In these reports, low levels of antibodies to HTLV-1 were present in a small proportion of inoculated animals, and this finding was regarded as evidence for HTLV-1 transmission. In our study, however, all orally infected rats lacked antibody responses. The discrepancy between our findings and those of previous studies may be due to differences in the species or experimental procedures. In such experiments, a minor injury during oral administration might cause a positive reaction. Further studies are required to clarify species differences.

Friedman and Weiner reported that the mechanism of oral tolerance induced by hen egg white lysozyme or myelin basic protein is determined by the dose of the antigen (3). A high dose induces anergy of antigen-specific Th1 cells, whereas a low dose induces active suppression mediated by regulatory T cells secreting suppressive cytokines (20–22, 40). In the present study, the exact dose of the antigen was difficult to evaluate, because the animals were persistently exposed to infecting HTLV-1 in addition to the initial dose of $5 \times 10^7$ MT-2 cells.

In the case of natural mother-to-child infection, the extent of exposure to HTLV-1 antigens may vary widely from one case to another. Moreover, HTLV-1-infected cells potentially produce cytokines affecting immune responses (12, 24, 34, 37, 38). Therefore, the HTLV-1 system may not be as simple as the hen egg white lysozyme or myelin basic protein system. Involvement of suppressive cytokines, such as transforming growth factor β or IL-10, and induction of certain active suppression mechanisms in the immune unresponsiveness against HTLV-1 remain to be clarified.

Interestingly, a number of intravenously or intraperitoneally inoculated rats demonstrated T-cell proliferation even without stimulation, as represented by the data for rat D6 (Fig. 4). A similar spontaneous lymphocyte proliferation has been reported for HTLV-1-infected individuals, particularly HAM/TSP patients (7, 19). This finding has been partly explained by the presence of an already activated HTLV-1-specific immune response in vivo and by HTLV-1-induced activation of growth factors and costimulatory molecules (18, 34). The absence of such spontaneous responses in orally inoculated animals in the present study suggested that host immune responses to HTLV-1 play a pivotal role in this phenomenon.

Many vertically HTLV-1-infected individuals lack antibody responses to HTLV-1 during infancy, suggesting that these subjects have, to a certain extent, immunological tolerance. Even among seroconverted individuals, the level of cellular immune responses to HTLV-1 varies and weak cellular immune responses are known to be associated with lymphoproliferative diseases. Our data for immune unresponsiveness against HTLV-1 not only in humoral but also in cellular immune responses emphasize the potential role of breast-feeding in early tolerance and weak cellular immune responses in some HTLV-1 carriers, which might be related to disease development. This possibility should be taken into consideration, particularly with prophylaxis of HTLV-1 infection.

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