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The genetic background as a determinant of human T-cell leukemia virus type 1 proviral load

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Abstract

Human T-cell leukemia virus type 1 (HTLV-1) is etiologically linked with HTLV-1-associated diseases. HTLV-1 proviral load is higher in persons with adult T-cell leukemia and HTLV-1-associated myelopathy/tropical spastic paraparesis than in asymptomatic carriers. However there are little data available on the factors controlling HTLV-1 proviral load in carriers. To study the effect of genetic background on HTLV-1 proviral load, we employed a mouse model of HTLV-1 infection that we had established. Here we analyzed nine strains of mice and found there is a great variation of proviral load among mouse strains that is not necessarily dependent on major histocompatibility complex. The antibody response is also different among these strains. To our knowledge, this is the first demonstration of the importance of the genetic background other than major histocompatibility complex controlling the HTLV-1 proviral load.

Keywords: HTLV-1; Proviral load; Genetic background; MHC; Mouse; Antibody; Carrier

Human T-cell leukemia virus type 1 (HTLV-1) is the first human retrovirus to be associated with malignancy. HTLV-1 has also been associated with the pathogenesis of non-malignant diseases such as HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP) and HTLV-1 associated uveitis (HAU). The proviral load in human peripheral blood mononuclear cells (PBMC) of HAM/TSP and HAU patients is reported to be higher than that in asymptomatic carriers [1,2], and it has been correlated with progression of motor disability in HAM/TSP [3]. The proviral load is also associated with likelihood of sexual transmission of HTLV-1 [4]. Therefore the level of HTLV-1 provirus in humans could be an important indicator of viral transmission and disease progression. In fact, the proviral load in PBMC in seropositive blood donors showed a 5 log difference [5]. However the factors determining the proviral load in asymptomatic carriers are not well understood. We hypothesized that the genetic background might play an important role in the determination of the proviral load in carrier conditions. To test this hypothesis, we employed an animal model of HTLV-1 infection. HTLV-1 can infect various species of animals [6–9], and we recently reported HTLV-1 transmission to newborn mice and clonal proliferation of HTLV-1-infected cells in the spleen [10–12].

The peripheral blood and the lymphoid organs are reported to constitute the major reservoirs for HTLV-1 [11–14]. We inoculated mice with the HTLV-1-producing human T-cell line, MT-2 cells [15,16], intraperitoneally in nine strains of syngeneic mice and measured the proviral load in the spleen and the peripheral blood by quantitative PCR. Special attention was made to evaluate any remaining inoculated cells in mice by cloning a MT-2 cell DNA sequence flanked to the 3′ LTR of HTLV-1 provirus and by establishing a sensitive PCR method. The results showed a great variation of proviral load in different strains of mice and suggested that the genetic background other than major histocompatibility complex is an important factor controlling HTLV-1 proviral load.

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Materials and methods

Cells and animals. HTLV-1-producing human T-cell line, MT-2 cells [15,16], and non-producer human T-cell line, ATL-1K cells [17], were cultured in RPMI 1640 supplemented with 10% fetal bovine serum. C57BL/6N, C57BL/6J, BALB/cAnN, BALB/cAnN-nu, DBA/2N, C3H/HeN, CBA/JN, and SJL/JOrlIco mice were obtained from Charles River, Tokyo, Japan, and C3H/HeJ mice were obtained from Clea, Tokyo, Japan. The mice were inoculated intraperitoneally with 2.5×10^6 MT-2 cells at the age of 4 weeks. To minimize any difference due to conditions of inoculated cells, MT-2 cells were collected into one lot of cell suspension and inoculated to all adult mice on the same day. The offspring of C57BL/6N, BALB/cAnN, C3H/HeN, and C3H/HeJ mice were also inoculated intraperitoneally with the same number of MT-2 cells within 24 h after birth. Peripheral blood samples were collected for determining anti-HTLV-1 antibody titers from all mice 3 weeks after inoculation. The mice were sacrificed 4 weeks after inoculation with ethyl ether anesthesia. The husbandry and experiments of all animals were conducted in line with the Regulations on Animal Experiments of University of Tsukuba and approved by the Animal Experiment Committee, University of Tsukuba.

DNA extraction. DNAs from the organs of the mice were prepared by sodium dodecyl sulfate–proteinase K digestion, followed by phenol extraction.

Quantification of HTLV-1 proviral load. The primer sets and probe for HTLV-1 *tax* region were as described previously [16]. We used mouse c-*myc* as an internal control. The primer sets were 5′-GAGCTGAA GCGCAGCTTTTT-3′, and 5′-GGCCTTTTCGTTGTTTTCCA-3′. A TaqMan probe was 5′-CCCTGCGTGACCAGATCCCTG-3′, which was covalently linked with a 5′-reporter dye, FAM (6-carboxy-fluorescein), to the 5′ end of the oligonucleotide, and with the 3′ quencher dye, TAMRA (6-carboxy-tetramethyl-rhodamine), at the 3′ end.

The pGEM-T easy vectors (Promega, USA) bearing a 3.3 kbp fragment encompassing parts of HTLV-1 *tax* or mouse c-*myc* sequences were used to establish standard curves for quantitative PCR. Plasmid DNAs containing from 10^0–10^4 *tax* molecules or 10^2–10^7 mouse c-*myc* molecules were mixed with 0.5 μg of λ phage DNA that did not contain viral component in 16.2 μl. The standard and mouse samples containing 0.5 μg of DNA were mixed with 8.8 μl of a PCR mixture. The reaction mixture in 25 μl contained 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 3.5 mM $MgCl_2$, 0.3 μM of each primer, 0.2 μM TaqMan probe, 200 μM dNTPs, and 1.25 U *Taq* polymerase (AmpliTaq Gold; Applied Biosystems, USA). The amplification of standard or sample DNAs was carried out in a 96-well reaction plate (Applied Biosystems, USA). All assays were performed in triplicate. The thermal cycler conditions were as follows: 95 °C for 10 min, then 50 cycles of 95 °C for 15 s and 60 °C for 1 min in case of *tax*: or 40 cycles of 95 °C for 15 s and 60 °C for 1 min in case of mouse c-*myc*. The DNAs from peripheral blood cells that did not show successful amplification of c-*myc* DNA were not used. We calculated the HTLV-1 proviral load per 1×10^5 mouse cells as (number of *tax* molecules/number of mouse c-*myc* molecules/2) $\times 10^5$.

Antibody detection. The antibodies against HTLV-1 proteins in the plasma were assayed with a particle agglutination kit (Serodia HTLV-1; Fujirebio, Japan).

Identification of the HTLV-1 integration site in MT-2 cell. To identify the flanking sequence of HTLV-1 integration site in MT-2 cells, we amplified a part of HTLV-1 3′ LTR and its flanking sequence by linker mediated PCR (LM-PCR), according to the procedure described by Fang J. et al. [10].

Detection of MT-2 specific sequences in DNA from mouse spleen. To assess any remaining MT-2 cells in mice, genomic DNA from spleen was subjected to PCR with a designed primer set to specifically amplify the human sequence flanked to 3′ LTR of HTLV-1 provirus. The set of primers for detection of a flanking sequence in MT-2 cells was 5′-CTGTTCTGCGCCGTTACAGATCGA-3′ (3′ LTR) and 5′-CATG AAGCATAAAATTCAAATTGT-3′ (the flanking sequence) (Fig. 1A). The reaction mixture in 25 μl contained 0.5 μg of sample DNA, 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 3.5 mM $MgCl_2$, 0.3 μM each primer, 200 μM dNTPs, and 0.1 U *Taq* polymerase (AmpliTaq Gold; Perkin–Elmer Applied Biosystems, USA). The thermal cycler conditions were as follows: 95 °C for 10 min, then 50 cycles of 95 °C for 30 s, 48 °C for 30 s, and 72 °C for 30 s, followed by a final stage of 72 °C for 7 min.

Statistical analysis. Welch's *t* test was used for statistical analysis.

Results

Difference of proviral load among mice with different genetic backgrounds

To examine the influence of genetic background on HTLV-1 proviral load, we compared the proviral load

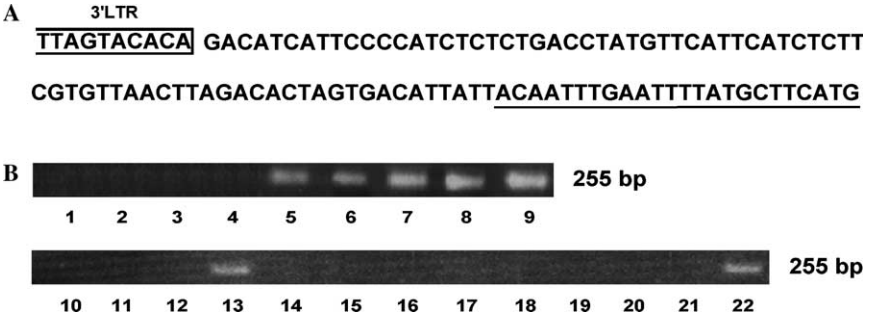

Fig. 1. A MT-2 cellular sequence flanked to 3′ LTR of HTLV-1 provirus and detection of MT-2 cell-specific sequence in mice by PCR. (A) A nucleotide sequence of MT-2 cells flanked to 3′ LTR of HTLV-1 provirus at the integration site was cloned and is shown. The position of the reverse primer used to detect MT-2 cell-specific flanking sequence is underlined. (B) Sensitivity of PCR for detecting MT-2 cell-specific sequences. In the sensitivity assay, DNAs from negative control cells and various numbers of MT-2 cells after serial dilutions with normal mouse spleen DNA were contained in each tube. The detection of remaining MT-2 cell-specific sequence in the spleen DNAs from various strains of mice was subjected to this PCR. A 255 bp fragment was amplified from MT-2 cells. The template DNAs of PCR were as follows (with the periods of MT-2 cell inoculation in parentheses): 1, Uninfected mouse spleen; 2, ATL-1K cells; 3 and 4, DNA equivalent to 0.3 MT-2 cell; 5 and 6, DNA equivalent to 1 MT-2 cell; 7 and 8, DNA equivalent to 3 MT-2 cells; 9, DNA equivalent to 10 MT-2 cells; 10, C57BL/6N (adulthood); 11, C57BL/6J (adulthood); 12, BALB/cAnN (adulthood, mouse 153); 13, BALB/cAnN-nu (adulthood); 14, DBA/2N (adulthood); 15, C3H/HeN (adulthood); 16, C3H/HeJ (adulthood); 17, DBA/JN (adulthood); 18, SJL/JOrlIco (adulthood); 19, C57BL/6N (newborn period); 20, BALB/cAnN (newborn period); 21, C3H/HeN (newborn period); and 22, C3H/HeJ (newborn period, mouse 162). The representative data are shown.

Table 1
The influence of genetic background on proviral load and antibody response

Mouse strain	H-2	Adult[a]			Newborn[a]		
		Proviral load[b]	Antibody titers	No. of mice	Proviral load[b]	Antibody titers	No of mice
C57BL/6N	b	95.3 ± 65.2	70 ± 35	5	29.1 ± 18.7	$<16 \sim 64$[e]	5
C57BL/6J	b	99.0 ± 21.2	224 ± 192	4			
BALB/cAnN	d	47.0 ± 19.1	410 ± 140	5	35.4 ± 19.1	$<16 \sim 128$[f]	4
BALB/cAnN-nu	d	0.6 ± 1.4	<16[c]	5			
DBA/2N	d	73.4 ± 22.0	179 ± 189	5			
C3H/HeN	k	154.7 ± 37.1	171 ± 74	3	5.5 ± 1.8	<16[c]	3
C3H/HeJ	k	35.0 ± 22.5	224 ± 64	4	8.2 ± 7.1	<16[c]	6
CBA/JN	k	6.2 ± 1.9	$<16 \sim 16$[d]	5			
SJL/JOrlIco	s	13.7 ± 6.2	1024 ± 725	4			

[a] The period of infection.
[b] Proviral load indicates the number of *tax* molecules in 10^5 spleen cells.
[c] Antibody titers in all mice were below the limit of detection.
[d] Antibody titers of 16 in two mice.
[e] Antibody titer of 64 in one mouse.
[f] Antibody titers of 64, 128, and 128 in three mice.

in the spleen among nine strains of mice. There were significant differences of proviral load among nine strains (Table 1). Statistical differences of proviral load among strains are shown as Table 2. Proviral load was statistically different between C3H/HeN and C3H/HeJ mice ($p < 0.05$) or C3H/HeN and CBA/JN mice ($p < 0.05$), even though they bore the same H-2^k haplotype. Among the strains that have H-2^d haplotype, proviral load values in BALB/cAnN and DBA/2N mice are not statistically different but they are significantly higher than that in BALB/cAnN-nu mice ($p < 0.01$).

We measured quantitatively the proviral load in the peripheral blood and compared it to that in the spleen among different strains of mice. The proviral load values in the peripheral blood of C57BL/6N, C57BL/6J, BALB/cAnN, DBA/2N, C3H/HeN, CBA/JN, and SJL/JOrlIco mice were 39.6 ± 10.4 (number of mice; $n = 4$), 32.4 ± 13.6 ($n = 3$), 13.0 ± 14.8 ($n = 5$), 49.8 ± 42.3 ($n = 3$), 61.3 ($n = 1$), 0 ($n = 1$), and 2.5 ± 3.3 ($n = 4$), respectively. The proviral load in the peripheral blood of all the mice examined was lower than that in the spleen of the corresponding mice. The mean values of proviral load in the peripheral blood and in the spleen were 31.3 ± 25.0 ($n = 21$) and 70.6 ± 38.5 ($n = 21$), respectively, and the difference was statistically significant ($p < 0.01$).

Effect of the period of HTLV-1 infection on proviral load

To assess the influence of the period of HTLV-1 infection and genetic background on proviral load, we also measured proviral load in mice infected within 24 h after birth (Table 1). Among four strains of mice infected at newborn period there were statistical differences in proviral load between BALB/cAnN and C3H/HeJ mice ($p < 0.05$). The proviral load in the mice infected at adulthood tended to be higher than that in the mice infected at newborn period. The differences of proviral load between mice infected at adulthood and at newborn period in C3H/HeN mice were statistically significant ($p < 0.05$).

Trace of inoculated MT-2 cells in mice

To trace any remaining MT-2 cells which should interfere with the evaluation of proviral load in infected mice, we performed LM-PCR and successfully cloned a sequence of MT-2 cell DNA flanked to 3′ LTR of HTLV-1 provirus (Fig. 1A). Then we made the primers to amplify the MT-2 cell-specific sequence, which was a part of the 3′ LTR of HTLV-1 provirus and its flanking sequence in MT-2 cells. We checked the sensitivity of PCR using a serial dilution of MT-2 cell DNA with normal mouse spleen DNA and detected MT-2 cell-specific sequence from a minimum of one MT-2 cell equivalent of DNA (Fig. 1B). Using this sensitive system we performed PCR with mouse spleen samples. We found that MT-2 cell-specific sequence could not be detected in most of the strains of mice, with the exception of one (mouse 153) out of five mice of BALB/cAnN-nu infected at adulthood and in three (mouse 162, 166, and 167) out of six mice of C3H/HeJ infected at newborn period (Fig. 1B). To trace the amount of MT-2 cells in these mice that showed positive signals, we performed this PCR in quadruplicate. In mouse 153, 162, 166, and 167, MT-2 cell-specific sequence was detected in one, four, three, and three out of four tubes, respectively. When these sample DNAs were diluted 5-fold for PCR in triplicate, MT-2 cell-specific sequences were detected in zero, one, zero, and zero out of three tubes, respectively.

Immune response to HTLV-1 infection

We measured antibody titers in the sera of mice inoculated with MT-2 cells at the age of 4 weeks and

Table 2
Difference of proviral load among mouse strains

Mouse strain	H-2	C57BL/6N	C57BL/6J	BALB/cAnN	BALB/cAnN-nu	DBA/2N	C3H/HeN	C3H/HeJ	CBA/JN	SJL/JOrlIco
C57BL/6N	b									
C57BL/6J	b									
BALB/cAnN	d	*	**							
BALB/cAnN-nu	d		**	**						
DBA/2N	d				**					
C3H/HeN	k			*	*	*				
C3H/HeJ	k		**			*	*			
CBA/JN	k	*	**	**	**	**	*			
SJL/JOrlIco	s	*	**	*	*	**	*			

Differences of proviral load with statistical significance are shown: *$p < 0.05$; **$p < 0.01$.

within 24 h after birth. The antibody titers ranged from <16 to 2048 (Table 1). There were significant differences of antibody titers among nine strains of mice infected at adulthood. The antibodies of mice infected at newborn period were detected from C57BL/6N (H-2^b) and BALB/cAnN (H-2^d) mice, but not from C3H/HeN and C3H/HeJ mice bearing H-2^k haplotype.

Discussion

We infected syngeneic inbred mice by inoculating MT-2 cells and demonstrated great differences of HTLV-1 proviral load among mouse strains, even within the same H-2 haplotype. Jeffery et al. [19] reported some association between MHC class I haplotype and HTLV-1 proviral load in healthy carriers. Therefore it is concluded that although the involvement of H-2 haplotype could not be excluded, non-H-2 genes are controlling HTLV-1 proviral load.

It was recently reported that C3H/HeJ mice show lower amplification of mouse mammary tumor virus than C3H/HeN mice [20]. C3H/HeJ mice have closely related genetic background to C3H/HeN mice, but have some defect in innate immunity due to a mutation of *Toll-like receptor 4* gene [21]. Our results that the proviral load of C3H/HeJ mice was significantly lower than that of C3H/HeN mice are consistent with an interesting hypothesis that innate immunity signaling genes are involved in determining HTLV-1 proviral load. Ohsugi reported the low detection rate of HTLV-1 provirus in HTLV-1-transformed rabbit T-cell line-inoculated C3H/He and C3H/HeJ mice and the detection rate between them was not significantly different [22]. The discrepancy might be due to the differences of HTLV-1-producing cells that are inoculated and the other experimental conditions. Low proviral load in BALB/cAnN-nu mice might be due to the absence of the microenvironment to support the growth of HTLV-1-infected cells [23].

Ishiguro et al. [7] reported that the detection rate of HTLV-1 provirus in four strains of rats infected during the newborn period was not different to that of rats infected at adulthood using qualitative PCR. We found that C57BL/6N, BALB/cAnN, C3H/HeN, and C3H/HeJ mice inoculated with MT-2 cells either at adulthood or at newborn period showed the provirus and there was no difference in the detection rate of HTLV-1 provirus. However, with quantitative PCR we showed that proviral load in mice infected at newborn period tended to be lower than that infected at adulthood (Table 1), and the difference in C3H/HeN mice was statistically significant ($p < 0.05$).

To exclude the possibility of any remaining inoculated MT-2 cells in mice, we cloned a part of cellular DNA sequence that flanked 3′ LTR of HTLV-1 provirus in MT-2 cells and established a sensitive PCR method. The results showed that the positive signals were only detected in one out of five BALB/cAnN-nu mice infected at adulthood and in three out of six in C3H/HeJ mice infected at newborn period. Therefore it is concluded that the inoculated MT-2 cells were rejected in most mice and that the viral load found in mice really represents the provirus present in the newly infected mouse cells. Concerning the mice that showed positive signals of MT-2 cell-specific sequence, we measured the number of MT-2 cells in mice by semiquantitative PCR method and calculated the number of *tax* molecules in those mice. It is calculated that about one third of the provirus in mice (nos. 153, 162, 166, and 167) could be due to the remaining MT-2 cells [16]. Previous reports indicated the involvement of NK cells in the rejection of MT-2 cells [24]. Since C3H/HeJ and BALB/c-nu mice carry congenital immune dysfunction [18,23,25], these mice might not be able to reject some of the inoculated MT-2 cells.

The peripheral blood and the lymphoid organs are reported to constitute the major reservoirs for HTLV-1 [11–14]. In the present work, it is interesting that the

provial load in the spleen is significantly higher than that in the peripheral blood irrespective of the genetic background of mice examined. This fact would be kept in mind in evaluating proviral load.

HTLV-1 carrier mouse models could be further used in analysis of the factors such as genes, chemicals, and environments to control HTLV-1 proviral load in vivo.

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