Journal of NeuroVirology (1999) 5, 414–420

http://www.jneurovirology.com

High incidence of meningeal infiltration by leukemic cells after infection of chimeric virus between neuropathogenic and non-neuropathogenic retroviruses

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Friend murine leukemia virus (F-MuLV) clone A8, previously shown to cause spongiform degeneration in rat brain, induced leukemia within 10 weeks after infection when inoculated into newborn rats. The chimeric virus Rec2, which contains the pol and env genes of 57 virus on the background of A8 and does not cause spongiform degeneration in the central nervous system (CNS), induced leukemic cell infiltration of the CNS, mainly of the meningeal region, in 58.3% of infected rats. In contrast, A8 induced little or no leukemic cell infiltration of the CNS. Other chimeric viruses containing the LTR and the 5′ half of the 5′ leader sequence of A8 induced aggressive leukemia, and after infection of these viruses, leukemic cell infiltration of the CNS was only observed in less than 20.0% of the rats. These results indicate that the fragment containing the LTR and the 5′ half of the 5′ leader sequence of A8 is essential for induction of aggressive leukemia in rats but is not sufficient to cause CNS infiltration. We found that leukemic cell infiltration of the CNS is dependent on the sequence of the virus.

Keywords: brain; paralysis; Friend virus; LTR; leukemogenesis; thymoma

Introduction

Clinical and pathogenic manifestations of leukemic involvement of the central nervous system (CNS) have been known for many years. The symptoms and signs resulting from leukemic involvement of the CNS are extremely varied. Neurologic complications in leukemia can manifest due to leukemic infiltration of the brain and cranial meninges or intracranial hemorrhages. A recent report proved that leukemic cell infiltration of the brain or spinal cord is commonly found in autopsies of neurologically asymptomatic patients with human chronic lymphocytic leukemia (Cramer *et al*, 1996). It is presumed that prolongation of survival by standard therapy against leukemia and lymphoma has been accompanied by an increase in the frequency of infiltration of the CNS, and that this complication might well occur during the late stage of leukemia, since there is a lack of neurological manifestation. On the other hand, some leukemic cell infiltration of the CNS can appear during the early phase of leukemia, even with neurological symptoms as an initial clinical sign (Bebin, 1968). The mechanism which leads to this predilection of leukemic infiltration of the CNS is not yet clear. Human T-cell lymphotropic virus type I (HTLV-I) infection induces either leukemia or HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP). In patients with the latter disease, the cerebrospinal fluid (CSF) contains anti-HTLV-I antibodies and may show a lymphocytic pleocytosis, as well as elevation in protein. Although HAM/TSP patients generally have normal lymphocyte numbers, morphologically atypical lymphocytes resembling ATL (adult T cell leukemia) cells can be seen in peripheral blood or in the CSF (Osame *et al*, 1987). The animal model obtained by the infection of HTLV-I in rats (Ishiguro *et al*, 1992) is not associated with perivascular cuffing with lymphocytes in the CNS, which is a characteristic neuro-pathological feature of HAM/TSP (Akizuki *et al*, 1987). In ecotropic mouse retrovirus infection, some viruses cause CNS infiltration by leukemic cells. Dawson *et al* (1966) reported that rats infected with one isolate, which was originally isolated by passage through rats, often develop hind-leg paralysis due to the infiltration by leukemic cells of the

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Received 8 June 1998; revised 5 April 1999; accepted 7 April 1999

vertebral column and meninges after slow development of leukemia. Portis *et al* (1990) reported that leukemic cell infiltration of the CNS after infection of weakly neurovirulent and highly leukemogenic mouse retrovirus correlates with expression of paralysis in mice. The responsible viral gene component still remains to be identified.

We have isolated FrC6 virus and its molecular clone A8 by serial passage of non-neuropathogenic Friend murine leukemia virus (F-MuLV) complex through newborn rat brain followed by long-term infection of a rat glial cell line (Watanabe and Takase-Yoden, 1995; Takase-Yoden and Watanabe, 1997). The FrC6 virus and its molecular clone, A8, is a replication-competent, ecotropic type C retrovirus which causes spongiform degeneration in the CNS of newborn rats without cell infiltration (Takase-Yoden and Watanabe, 1997). During the course of investigation of genes of A8 responsible for its pathogenicity using chimerae between A8 virus and non-neuropathogenic 57 virus, we found a chimeric virus which frequently induces leukemic cell infiltration of the CNS.

In the present study, we attempted to map the genetic determinant(s) responsible for CNS tropism of leukemic cell infiltration using chimeric viruses combining A8 and 57. The studies showed that the primary determinant for induction of leukemia and thymoma in rats lies in the LTR and the 5′ half of the 5′ leader sequence of A8. Furthermore, the chimeric virus containing the pol and env genes of 57 in addition to the LTR and the 5′ half of the 5′ leader sequence of A8 caused leukemic cell infiltration of meninges more frequently than the other leukemogenic chimerae. These results indicate that infiltration of the CNS by leukemic cells is dependent on the sequence of the virus.

Results

Infiltration of the CNS by leukemic cells after infection of the chimerae between A8 and 57

Among the rats infected with A8, 57, or their chimeric viruses, infiltration of the CNS by leukemic cells was observed in A8, Rec2, Rec6, and Rec7-infected rats at 6–10 weeks post-infection (Figure 1). The structure of chimeric viruses is presented in Figure 2. The highest incidence of CNS infiltration was 58.3% (n=12) of Rec2-infected rats. Other viruses caused CNS infiltration with incidences ranging from 10.5–20.0%. In some rats infected with these viruses, hind-leg paralysis or weakness was observed (Table 1). In some infected rats, infiltration of brain (brain hemisphere, cerebellum, and brain stem) was observed, but infiltration of spinal cord was not detected in any rats. In the brains of Rec2-infected rats, leukemic cells were mainly observed in the meninx, but the cortex and the white matter, mainly in Virchow-Robin spaces, were also involved (Figure 3A).

As shown in Figure 1, A8, Rec2, Rec6, Rec7, and R7a induced leukemic cell infiltration of organs other than the CNS and thymus at 6–10 weeks post-infection with a high incidence, ranging from 60.0–100% of the infected rats. Thymoma was also

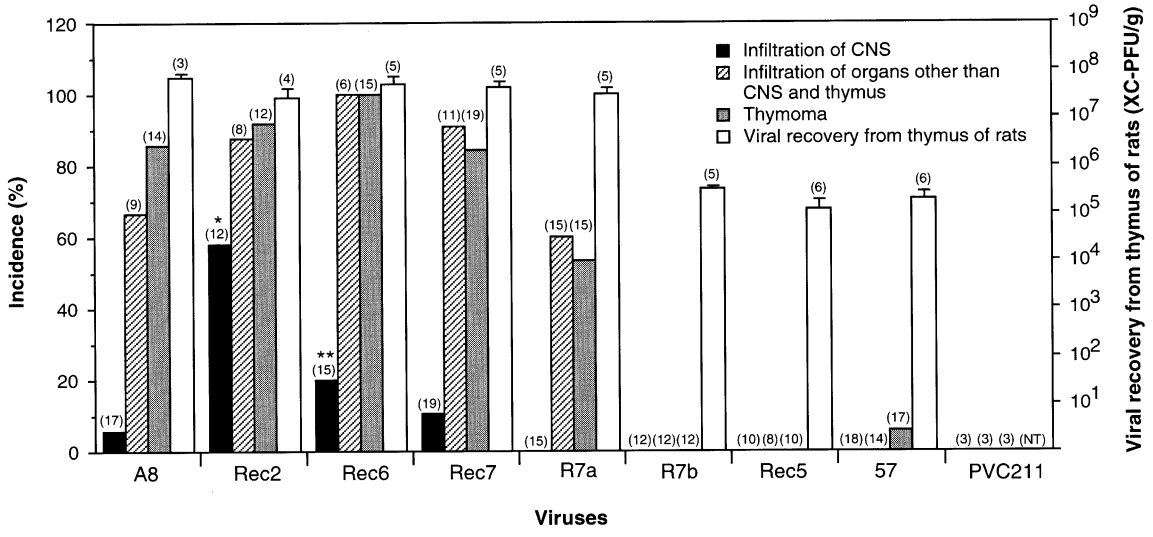

Figure 1 Incidence of leukemic cell infiltration of rat organs and thymoma. Viruses were inoculated intraperitoneally and intracerebrally into newborn Lewis rats (A8: $8.0\times10^4-3.6\times10^5$ XC-PFU/rat. Rec2: $7.1\times10^5-3.8\times10^6$ XC-PFU/rat. Rec6: 2.0×10^5 XC-PFU/rat. Rec7: 2.4×10^6 XC-PFU/rat. R7a: 6.4×10^4 XC-PFU/rat. R7b: 4.4×10^4 XC-PFU/rat. Rec5: $4.6\times10^3-1.1\times10^5$ XC-PFU/rat. 57: $1.8\times10^4-9.3\times10^4$ XC-PFU/rat. PVC211: 2.4×10^4 XC-PFU/rat.). Six to 10 weeks later, leukemic cell infiltration of the organs and thymoma was evaluated. The numerals enclosed in parentheses represent numbers of rats. Virus titers in the thymus were determined by XC plaque assay. Means and s.e.m. are shown. Statistical comparison was performed by the χ squared test. There was no significant difference between the incidences of infiltration of the organs other than the CNS and thymus by A8, Rec2, Rec6, Rec7, or R7a. *$P<0.01$ *vs* A8, $P<0.025$ *vs* Rec7. **Difference is not significant compared to A8 or Rec7.

caused by these viruses with incidences ranging from 53.3–100%. In contrast, 57 virus and the chimeric viruses R7b and Rec5, which do not contain the LTR and the 5′ half of the 5′ leader sequence of A8 origin, showed little tumorgenicity or leukemogenecity. Neither leukemic cell infiltration of the organs nor thymoma was observed in the rats infected with PVC211, which is a neuropathogenic variant of F-MuLV and closely related to A8 (Figure 1). Virus recovery from thymus was measured. Virus titers of A8, Rec2, Rec6, Rec7, and R7a-infected rats were of about the same level (Figure 1). Virus titers of the thymus of R7b, Rec5, and 57-infected rats were lower than those of A8, Rec2, Rec6, Rec7, and R7a-infected rats. As shown in Figure 4, rats injected with Rec2, Rec6 or Rec7 died with the same latency as those infected with A8, whereas rats injected with Rec5 and R7b died 10 weeks later, around the same time as those treated with 57. Therefore, only the *Cla*I-*Aat*II fragment of A8, containing the LTR and the 5′ half of the 5′ leader sequence, is essential for causing aggressive leukemia of which infected rats die with a short latency. In contrast, induction of infiltration of the CNS requires the region containing the pol and env

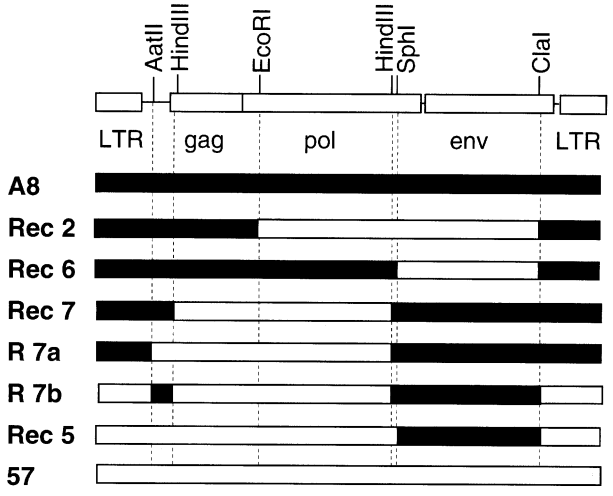

Figure 2 Structure of chimeric viruses derived from A8 and 57. The viral genomes are represented in nonpermutated form, flanked at their ends by the LTR sequence. In recombinant viral genomes, solid regions are sequences derived from the A8 virus and open regions are sequences derived from the 57 virus.

Table 1 Correlation between clinical expression of neurological disease and leukemic cell infiltration

Virus[a]	*Hind-leg paralysis or weakness*[b]	*Leukemic cell infiltration of*[b] *Brain*	*Spinal cord*
A8*	52.3 (21)	5.9 (17)	0 (12)
Rec2	71.4 (14)	58.3 (12)	0 (11)
Rec6	26.7 (15)	20.0 (15)	0 (6)
Rec7*	66.7 (12)	10.5 (19)	0 (11)

[a]Newborn Lewis rats were inoculated with A8, Rec2, Rec6, or Rec7. The inocula are described in the legend to Figure 1. [b]Percentage of rats which manifested clinical expression or pathological changes. The numerals enclosed in parenthesis represent numbers of inoculated rats. *Spongiform degeneration was observed in CNS of all infected rats, as described previously (Takase-Yoden and Watanabe, 1997).

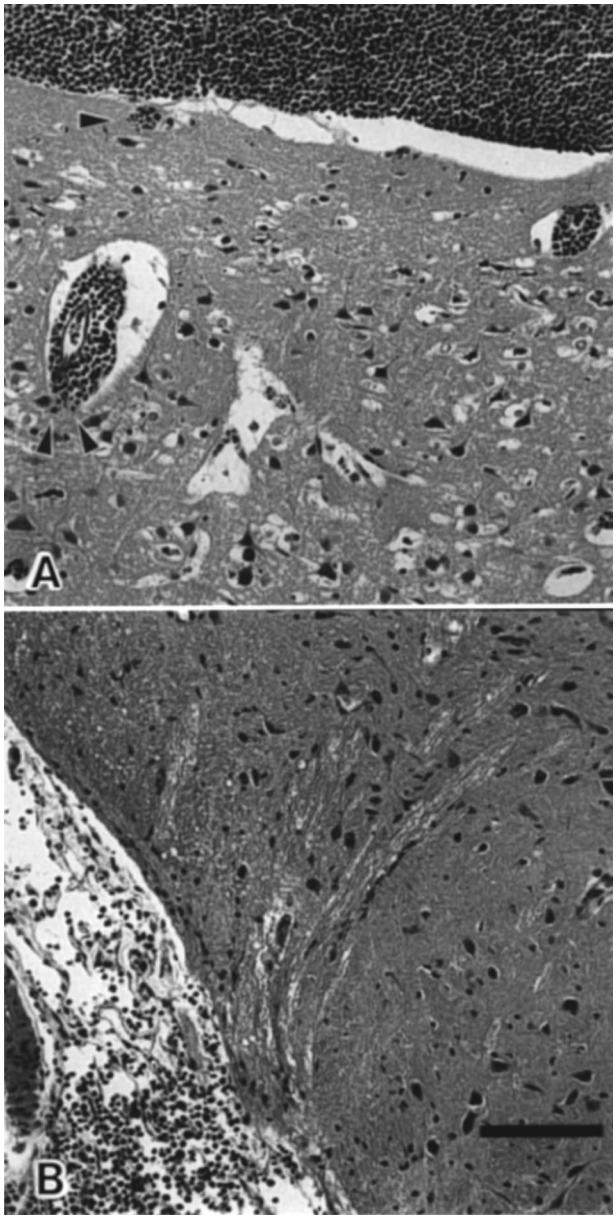

Figure 3 Leukemic cells infiltrated the CNS of rats infected with Rec2 (**A**) and A8 (**B**) virus. (**A**) Blastic lymphoid cells occupy the meningeal space. The infiltrates extend to the brain cortex. Note that the leukemic cells infiltrate beyond the Virchow-Robin spaces (arrowheads). (**B**) Leukemic cell infiltration of the CNS was detected in one of 17 rats infected with A8. The infiltration was of minimal degree: it involved only a few blastic cells and was restricted to the meningeal space. Hematoxylin-eosin staining of a paraffin-embedded section. Bar indicates 100 micrometers.

genes of 57 in addition to the LTR and the 5′ half of the 5′ leader sequence of A8.

To investigate the leukemogenicity and tumorgenicity of A8, 57, and PVC211 in mice, we injected virus into newborn BALB/c mice. Splenomegaly was induced in all mice (n=9 and 7, respectively) within 2–3 months (Table 2). In the mice infected with PVC211, splenomegaly was induced at 5–9 months post-infection in 43% (n=7) of the animals.

Correlation between leukemic cell infiltration of CNS and phenotype of leukemia/lymphoma

To study the correlation between leukemic cell infiltration of CNS and the degree of leukemic cell infiltration of general organs, the leukemic cell infiltration of each organ was scored (Table 3). The degree of infiltration caused by Rec6-infection was highest. There was a significant difference between the degree of infiltration caused by Rec6-infection and those caused by A8-infection ($P<0.05$), Rec7-infection ($P<0.05$) or R7a-infection ($P<0.02$). The degree of infiltration caused by Rec2-infection was comparable with those caused by A8-infection, Rec7-infection, or R7a-infection.

The kinetics of leukemic cell infiltration and induction of thymoma by Rec2 and A8 were investigated. As shown in Figure 5A, at 4 weeks post-infection, leukemia was observed in 40.0% of Rec2-infected rats, but the CNS was not infiltrated by leukemic cells. Infiltration of the CNS by leukemic cells in Rec2-infected rats was observed at 6–7 weeks post-infection. At 8–10 weeks post-infection, 100% of Rec2-infected rats manifested leukemia and thymoma. At this time, the incidence of CNS infiltration by leukemic cells reached 66.7% in Rec2-infected rats. Although leukemia induced by A8 was detected later than that induced by Rec2, 100% of A8-infected rats manifested leukemia and thymoma at 8–10 weeks post-infection (Figure 5B). At that time, CNS infiltration by leukemic cells was not detected. At 6–7 weeks post-infection of the A8 virus, only one rat exhibited leukemic cell infiltration of the CNS. This infiltration was slight, contained few blastic lymphocytes and was restricted to the meninx (Figure 3B). The kinetic curve of incidence of thymoma in Rec2-infected rats was the same as that in A8-infected rats.

Table 3 The degree of leukemic cell infiltration of rat organs other than CNS and thymus

Virus[a] *(Number of rats)*	*Number of rats which manifested leukemic cell infiltration of organs other than CNS and thymus*[b] *Grade 0*	*Grade I*	*Grade II*	*Grade III*	*Score*[c]
A8 (9)	3	3	1	2	1.22±0.41
Rec2 (8)	1	2	4	1	1.63±0.32**
Rec6 (6)	0	1	1	4	2.50±0.34*
Rec7 (11)	1	6	3	1	1.36±0.24
R7a (15)	6	4	2	3	1.13±0.31

[a]Newborn Lewis rats were inoculated with A8, Rec2, Rec6, Rec7, or R7a. The inocula are described in the legend to Figure 1. The numerals enclosed in parentheses represent numbers of inoculated rats. [b]Grade 0: no leukemic cell infiltration, grade I: mild infiltration of one or two organs, grade II: strong infiltration of one or two organs or mild infiltration of more than three organs, grade III: strong infiltration of more than three organs. [c]The intensity of leukemic cell infiltration is expressed by numbers for statistical comparison. The sum of the grade (grade 0: 0, grade I: 1, grade II: 2, grade III: 3) of each animal was divided by the number of inoculated animals. Means and s.e.m. are indicated. *$P<0.05$ *vs* A8 or Rec7, $P<0.02$ *vs* R7a, using the *t*-test. **Difference is not significant compared to A8, Rec7, or R7a according to the *t*-test.

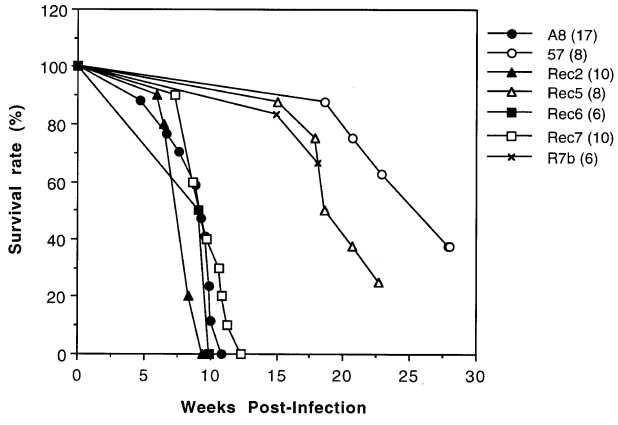

Figure 4 Survival rate of rats infected with A8, 57, and their chimerae. Viruses were inoculated intraperitoneally and intracerebrally into newborn Lewis rats. The inocula are described in the legend to Figure 1. The numerals enclosed in parentheses represent numbers of rats.

Table 2 Incidence of splenomegaly in mice

Infected with[a]	*Incubation period (months)* 2–3	5–9
A8	100% (9)[b]	NT[c]
57	100% (7)	NT
PVC211	0% (3)	43% (7)

[a]Newborn BALB/c mice were inoculated intraperitoneally with A8 (five mice, 4.3×10^4 XC-PFU/mouse, four mice, 8.5×10^5 XC-PFU/mouse), 57 (2.1×10^4 XC-PFU/mouse), or PVC211 (1.2×10^4 XC-PFU/mouse). [b]The numerals enclosed in parentheses represent numbers of mice. [c]Not tested.

Discussion

The incidence of leukemic cell infiltration of the CNS, mainly meninges, was found to be higher with Rec2 than with A8 or Rec7 infection ($P<0.01$ and $P<0.025$, respectively), although all of these viruses caused leukemia and thymoma to similar extents (Figure 1). Furthermore, the infiltration of the CNS observed after A8 infection was slight, involving

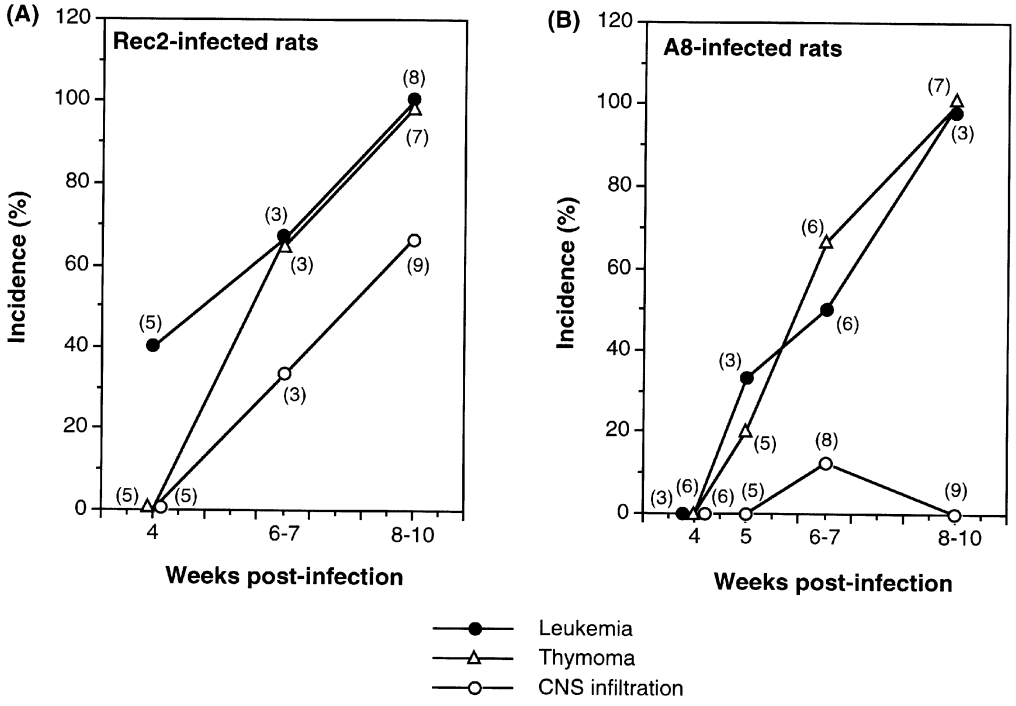

Figure 5 Kinetics of leukemic cell infiltration of rat organs and thymoma. Viruses were inoculated intraperitoneally and intracerebrally into newborn Lewis rats. The inocula are described in the legend to Figure 1. The numerals enclosed in parentheses represent numbers of rats.

only a few blastic lymphocytes, and was restricted to the meninx (Figure 3B). The degree of infiltration of organs other than the CNS by Rec2 was also the same as that by A8, Rec7, and R7a (Table 3). Furthermore, Rec6 exhibited the same rate of CNS involvement of leukemia as A8 and Rec7 (Figure 1), although Rec6 caused more extensive leukemic infiltration of other organs than did A8 ($P<0.05$) and Rec7 ($P<0.05$) (Table 3). These results indicate that the high incidence of infiltration of the CNS after Rec2 infection was dependent on the viral sequence rather than on the general intensity of leukemic infiltration. Therefore, the findings suggest that the regions containing the LTR and the 5′ half of the 5′ leader sequence of A8, 57-pol and env are responsible for leukemic cell infiltration of the CNS.

We investigated the genetic determinants of leukemogenicity and tumorgenicity in rats. The 57 virus, Rec5, and R7b did not show leukemogenicity or tumorgenicity in rats within 10 weeks of infection. The A8 virus, Rec2, and Rec6 induced leukemia and thymoma in that time period. Therefore, the *Eco*RI-*Cla*I fragment containing the pol and env genes of A8 is not the primary determinant for induction of aggressive leukemia/thymoma. Rec7 induced leukemia and thymoma to the same extent as A8, indicating that the determinants for induction of aggressive leukemia/thymoma do not reside in the *Hind*III-*Hind*III fragment containing the gag and pol genes of A8. R7a induced leukemia and thymoma at 6–10 weeks post-infection but R7b did not. These results indicate that the primary determinants for induction of aggressive leukemia/thymoma in rats reside in the *Cla*I-*Aat*II fragment of A8 containing the LTR and the 5′ half of the 5′ leader sequence. This region of A8 is sufficient to induce aggressive leukemia, but the region containing pol and env of 57 was also necessary for causing leukemic cell infiltration of CNS.

A major difference between A8-LTR and 57-LTR was a shift of the second nuclear factor binding site, FVa, of A8 to FVb2/NF1 in 57-U3 (Manley *et al*, 1989; Takase-Yoden and Watanabe, 1997). The importance of the repeat of FVa in A8-U3 for causing tumorgenicity/leukemogenicity in rats was also ascertained by comparison of this region with the U3 sequence of PVC-211 (Masuda *et al*, 1992), which has very low tumorgenicity/leukemogenicity in rats (Figure 1; Kai and Furuta, 1984). The LTR of PVC211 lacks two FVa sites compared with A8-U3. This difference could be responsible for the aggressive leukemogenicity of A8 in rats. In terms of tumorgenicity/leukemogenicity in mice, the A8 and 57 viruses shared almost the same efficiency of inducing splenomegaly and erythroleukemia, but PVC211 had very low efficiency (Table 2). These results indicate that besides the function of each

nuclear factor binding site, the number of nuclear factor binding sites or length of the direct repeat region could contribute to the short incubation period for induction of erythroleukemia in mice.

A time course study revealed that all rats infected with A8 and Rec2 manifested leukemia and thymoma at 8–10 weeks post-infection, whereas leukemic cell infiltration of the CNS was not caused by A8 during this period. The leukemic cell infiltration of the CNS after Rec2-virus infection occurred later than the infiltration of the other organs. Primarily, transformation of the cells occurred in hematogenous tissues and/or thymus, and thereafter leukemic cells infiltrated the CNS. These results suggest that the leukemic cells induced by Rec2 and A8 might differ in some biological character(s), such as adhesion molecule interaction or chemokine responsiveness. A8 and Rec2 may infect different types of lymphocytes that may or may not enter the CNS. Comparison of the biological activities of the leukemic cells induced by Rec2 and A8 is now in progress using leukemic cell lines established from rats infected with these viruses. Another possibility is that the virus itself does not determine the phenotype of leukemic cells, but rather that it affects other factors that lead to CNS infiltration; for example, it might alter the function of CNS endothelial cells or glial cells by interaction of the virus-derived env protein with some molecule(s) on these cells (Takase-Yoden and Watanabe, 1999). In the brains of rats infected with A8 or Rec7, strong gliosis was manifested, accompanied by spongiform neurodegeneration (manuscript in preparation). In these rats, the incidence of leukemic cell infiltration of the CNS was very low. The presence of glial cell activation might block extensive infiltration of the CNS by leukemic cells.

The A8 and Rec7 viruses induced spongiform degeneration in the brain in all infected animals, whereas spongiform lesions were only weakly apparent in spinal cords (Takase-Yoden and Watanabe, 1997). The A8 and Rec7 viruses caused hind-leg paralysis or weakness in 52.3 and 66.7% of infected rats, respectively (Table 1). PVC211 induces strong spongiform degeneration in the spinal cord (Kai and Furuta, 1984; Hoffman *et al*, 1992; Takase-Yoden and Watanabe, 1997), and almost all rats infected with PVC211 showed hind-leg paralysis or weakness. These results indicate that in spongiform degeneration of the CNS, there is a correlation between the clinical expression and the distribution and intensity of lesions. The Rec2 and Rec6 viruses caused hind-leg paralysis or weakness in 71.4 and 26.7% of rats, respectively, although Rec2 and Rec6 did not induce spongiform degeneration in the CNS or leukemic cell infiltration of the spinal cord (Table 1; Takase-Yoden and Watanabe, 1997). Portis *et al* (1990) reported that paralysis occurred through infiltration of the spinal cord by leukemic cells after infection by weakly neurovirulent and highly leukemogenic virus. In the present study, there was no direct correlation of infiltration of the spinal cord by leukemic cells and clinical expression. In Rec2 and Rec6 infection, leukemic cell infiltration was observed mainly in meningeal and Virchow-Robin spaces of the brain, and not in spinal cord (Table 1). Therefore, the high incidence of hind-leg paralysis or weakness caused by Rec2 or Rec6 infection compared with A8 infection might result from leukemic cell infiltration of these regions.

Materials and methods

Viruses and cells

Neuropathogenic FrC6 virus clone A8 was obtained as described previously (Takase-Yoden and Watanabe, 1997). The infectious DNA of clone 57 of F-MuLV (Oliff *et al*, 1980) and A8 was transfected into NIH3T3 cells, and a virus-producing cell culture was established. Chimeric viruses combining A8 and 57 were prepared as described previously (Takase-Yoden and Watanabe, 1997). Basic recombinant DNA procedures were performed according to standard methods (Sambrook *et al*, 1989). PVC211-producing normal rat kidney cells (NRK) were provided by Dr K Kai, Yamaguchi University, Yamaguchi, Japan. The supernatants of these cells were used to infect NIH3T3 cells, and the supernatants of virus-producing cultures were used in the experiments. Viral titers were determined by XC cell plaque assay of C182 cells in the presence of 10 μg/ml of Polybrene (Rowe *et al*, 1970). The cells, except for C182, were grown on Dulbecco's modified Eagle's medium containing 10% fetal calf serum. C182 cells were grown on minimum essential medium supplemented with 10% calf serum.

Animals

The ability of viruses to cause disease was assessed by using newborn Lewis rats and BALB/c mice purchased from a commercial breeder. Newborn rats were inoculated intraperitoneally with 0.1 ml and intracerebrally with 0.005 ml of viral supernatant. Newborn mice were inoculated intraperitoneally with 0.05 ml of viral supernatant. Titers of inoculated virus are described in the figure legends.

After exanguination, thymuses of rats were homogenized in cold phosphate buffered saline (PBS) containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$, and infectious virus titers were determined by the XC cell plaque assay (Rowe *et al*, 1970).

Histology

The organs of the animals were immersed in 4% paraformaldehyde buffered with 0.12 M phosphate (pH 7.3) and fixed. Some rats were perfused with

4% paraformaldehyde in 0.12 M phosphate buffer (pH 7.3). The tissues were embedded in paraffin for histological staining with hematoxylin and eosin.

Nucleotide sequence accession number
The DDBJ, EMBL, and GenBank accession number of the sequence in this report is D88386.

Acknowledgements

The authors thank Keiko Wakabayashi for measuring virus titers, and Katsumi Goto for performing histology. This work was supported in part by a grant from the Science and Technology Agency of Japan (13073-2125-14).

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