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Two Major Subgroups of Human T-Cell Leukemia Virus-1 in Japan

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Abstract

T lymphocytes of patients with human T-cell leukemia virus type 1 (HTLV-1)-associated myelopathy (HAM) were cultured. After cultivating for several months, HAM-derived cell lines were tested for the presence of HTLV-1 proviral genome. We have found two major subgroups, the *Sac*I type and the *Pst*I type, of HTLV-1 by the restriction map analysis. They were almost equally distributed among HAM patients. We have also found two types of the provirus in DNA derived from fresh peripheral blood lymphocytes (PBL) or lymph node cells of adult T-cell leukemia/lymphoma (ATL) patients. The *Pst*I type proviruses were predominant in ATL patients. It was concluded that two major subgroups of HTLV-1 exist in Japan and both types have an ability to cause either of two diseases, ATL or HAM.

Introduction

Human T-cell leukemia virus type 1 (HTLV-1) is an exogenous retrovirus associated with an endemic human T-cell malignancy termed adult T-cell leukemia/lymphoma (ATL) (1–3). We examined the state of HTLV-1 provirus in leukemic cells of ATL patients (4), and demonstrated that all the T-cell clones proliferating in a tumor can harbor HTLV-1 provirus with one to three copies or with defective proviral copies in some cases. Recently a neurological disease termed HTLV-1-

associated myelopathy (HAM) has been reported among HTLV-1 carriers (5,6). The reason that the same virus causes different diseases like ATL and HAM remains to be elucidated. Although viruses detected in HAM and ATL are shown to be identical in DNA blotting (7), the presence of genomic variations among HTLV-1 isolated from ATL have been reported (8–10). In order to elucidate a possible relationship between clinical manifestations and virus subtypes, we investigated the restriction enzyme sites of HTLV-1 proviruses from patients with ATL or HAM.

Materials and methods

Cell lines

Human T-cell lines derived from healthy HTLV-1 carriers were established as described previously (11). To establish HAM cell lines, mononuclear cells were separated by Ficoll-Hypaque centrifugation from heparinized samples of the patients' peripheral blood. Cells were cultured in RPMI 1640 medium supplemented with 20% fetal bovine serum in the presence of crude interleukin-2 (IL-2). Conditioning medium containing IL-2 was prepared by culturing mitogen-stimulated peripheral blood lymphocytes and spleen cells as described (12).

DNA extraction and Southern blot hybridization

Fresh tumor cells were collected from peripheral blood and separated by Ficoll-Hypaque density-gradient centrifugation, or from lymph nodes by mincing and elimination of aggregates after sedimentation. Preparation of high molecular weight DNA from cells and procedure of Southern blot hybridization were described previously (4,13). Briefly cells were lysed in STE (0.1 M NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA), 0.5% $NaDodSO_4$, 500 μg/ml proteinase K. After incubating at 37°C for 1 hr, lysates were extracted with 3 × STE-saturated phenol and the aqueous phase was ethanol precipitated. Precipitates were washed once with cold 70% ethanol, air dried, and then dissolved in water. DNA was then treated with RNase (100 μg/ml) at 37°C for 45 min followed by proteinase K digestion (500 μg/ml) at 37°C for 45 min. DNA was extracted again with 3 × STE-saturated phenol and precipitated with ethanol. DNA thus obtained was dissolved in TE (10 mM Tris-HCL, pH 7.4, 1 mM EDTA) and stored at 4°C.

DNA (3μg) completely digested with *Sac*I or *Pst*I was separated on 0.7% agarose gel. After electrophoresis DNA on the gel was transferred to a nitrocellulose membrane (Schleicher & Schuell, W. Germany). *Hind*III-digested λ DNA was used as a molecular size marker.

The HTLV-I probe used was a mixture of a 1 kb *Sma*I fragment (gag sequences), a 2 kb *Hind*III fragment (pol sequences), a 0.4 kb *Bam*HI-*Xho*I fragment (env se-

quences) and a 0.8 kb *Pst*I-*Cla*I fragment (pX sequences) from the λ phages containing the entire or defective HTLV-I proviruses (10,13). The ^{32}P-labeled nick-translated probes were prepared using a nick-translation kit purchased from Amersham (Amersham, Japan). [^{32}P]dCTP (NEN; 600 Ci/mmol) was used for labeling DNAs. After hybridization, filters were washed in 15 mM NaCl, 1.5 mM sodium citrate, 0.5% $NaDodSO_4$ at 50°C. Filters were autoradiographed with intensifying screen for 2–7 days at −70°C. Restriction endonucleases were purchased from Takara Shuzo Co. Kyoto, Japan.

Results

Southern blot hybridization of DNA extracted from T-cell lines established from peripheral blood lymphocytes (PBL) of HAM patients

T-cell lines were established from PBL of 15 patients with HAM. High molecular weight DNA derived from each HAM cell line was examined for the presence of HTLV-1 provirus by Southern blot hybridization using the specific fragments of HTLV-1 genome as the probe. The restriction endonuclease *Sac*I cuts in the long terminal repeat (LTR) of the HTLV-1 isolated from cell line MT-2, thereby generating an 8.5 kb internal fragment which corresponds to the complete proviral genome as described previously (10). Therefore the restriction enzyme *Sac*I was used to cut DNA from the primary T-cell lines established from PBL of 15 HAM patients (lanes 1–15 in Fig. 1). A conspicuous band of 8.5 kb in lanes 1, 4, 9, 11, 12, 14, and 15 in Fig. 1 indicates that, like an MT-2 type HTLV-1, *Sac*I cuts in the LTR of the proviruses in 7 of 15 cell lines established from PBL of the

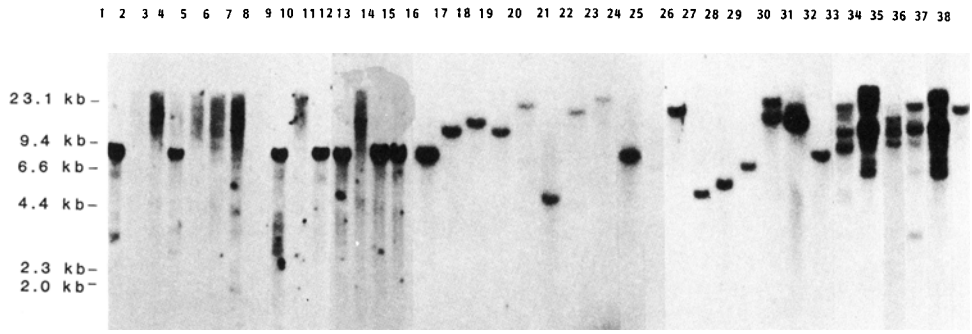

Fig. 1. Southern blot of DNA treated by *Sac*I restriction endonuclease with HTLV-1 probe. Specific fragments of HTLV-1 described in Materials and Methods were hybridized with *Sac*I-digested DNA prepared from different cell lines or ATL tumor clones. Lanes 1 to 5 are DNA derived from HAM cell lines, and lanes 16 to 32 are DNA derived from fresh peripheral blood lymphocytes or lymph node cells of ATL patients, and lanes 33 to 38 are DNA of cell lines established from healthy carriers of HTLV-1.

HAM patients. In the other 8 lanes (lanes 2, 3, 5, 6, 7, 8, 10, and 13 in Fig. 1), however , *Sac*I digestion gave smears but not any discernible discrete band, suggesting the lack of the site of this enzyme within these proviruses.

Next, *Pst*I was used to cut the same samples. *Pst*I, which yields characteristic internal bands in all known HTLV-1 isolates, gave several discrete bands of HTLV-1 genome in DNA derived from all HAM cell lines as shown in Fig. 2 (lanes 1 to 15). Among the detectible bands, a unique band of 0.5 kb was observed in 8 lanes (lanes 2, 3, 5, 6, 7, 8, 10, and 13 in Fig. 2). When the 5′pX portion was used as a single probe, this band was detected in the same 8 lanes (data not shown). These results indicate that *Pst*I cuts in the 5′pX region of the only provirus whose LTR has no *Sac*I restriction site. The presence of either the 8.5 kb *Sac*I fragment or the 0.5 kb *Pst*I fragment is mutually exclusive. Thus these results led us to conclude that the proviruses in PBL of HAM patients can be classified into two major subtypes by the restriction map analysis, i.e., *Sac*I type without 0.5 kb band in *Pst*I digestion and *Pst*I type without 8.5 kb band in *Sac*I digestion.

The only exception is the cell line 4 which has a *Sac*I subgroup of HTLV-1 with a very faint band of 0.5 kb by the *Pst*I treatment (lane 4 of Figs. 1 and 2). It is most probable that the cell line contains the mixture of major *Sac*I and minor *Pst*I type HTLV-1.

Southern blot hybridization of DNA extracted from periferal blood lymphocytes or lymph node cells of ATL patients and T-cell lines established from peripheral blood lymphocytes of healthy HTLV-1 carriers

The same procedure was performed on fresh samples derived from ATL patients (lanes 16 to 32 in Figs. 1 and 2) and also on cell lines established from healthy HTLV-1 carriers (lanes 33 to 38 in Figs. 1 and 2).

Among 17 ATL patients screened, *Sac*I yielded a single band of 8.5 kb in 3 cases

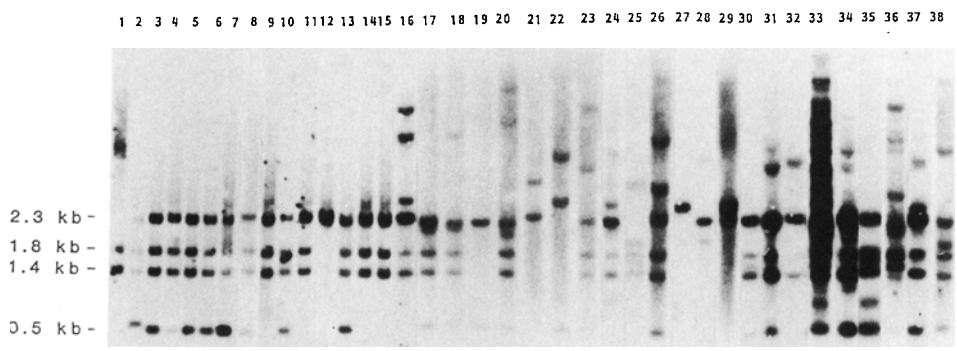

Fig. 2. Southern blot of DNA treated by *Pst*I restriction endonuclease. Lane numbers are the same as described in Fig. 2.

(lane 16, 24, and 32 in Fig. 1), while *Pst*I digestion gave a characteristic band of 0.5 kb in 9 cases (lane 17, 18, 19, 20, 22, 23, 26, 30, and 31). The multiple high molecular weight bands other than 2.3, 1.8 and 1.4 kb by *Pst*I treatment are probably junction fragments with cellular DNA. Thus this result indicates that the proviruses in ATL patients can be classified into two subgroups like the proviruses in HAM patients and that the *Sac*I type HTLV-1 appears to be fewer than the *Pst*I type. We also observed several defective type HTLV-1 in these samples. After *Sac*I treatment, the bands smaller than 8.5 Kb in 5 lanes (lanes 21, 25, 27, 28, and 29) suggest that these cell lines contain a defective provirus of HTLV-1, respectively.

Though the available cell lines (lanes 33 to 38) derived from healthy carriers of HTLV-1 are limited in number, all of them are of the *Pst*I type from the analysis of *Sac*I or *Pst*I digestion (Figs. 1 and 2).

Discussion

The results presented here are summarized in Fig. 3 and Table 1. They indicate that two major subgroups of HTLV-1, namely *Sac*I and *Pst*I types, exist in Japan and both have an ability to cause ATL or HAM. Among HAM patients, two subgroups of HTLV-1 were almost equally distributed. The main clinical manifestations of HAM were spastic paraparesis with pyramidal signs, urinary disturbance, and slight sensory disturbances (6). The viruses analyzed here were from peripheral lymphocytes, not cerebrospinal fluid. It is reasonably assumed that HAM patients established their infection of HTLV-1 once in a life time, thus causing HAM by the same group of HTLV-1 shown in their blood lymphocytes. To verify this assumption, characterization of the virus, if it is found in spinal lesions, will be important. Though the *Pst*I type was predominant among the ATL patients in this examination, more extensive studies will be needed to verify the deviation of any particular subgroup of HTLV-1 with ATL leukemogenesis. The ATL samples were gathered from different areas of Japan including the southwestern part (Okinawa, Nagasaki, and Uwajima), central part (Kyoto, Mie, and Osaka) and northern part (Akita). Two subgroups of HTLV-1 were relatively well distributed

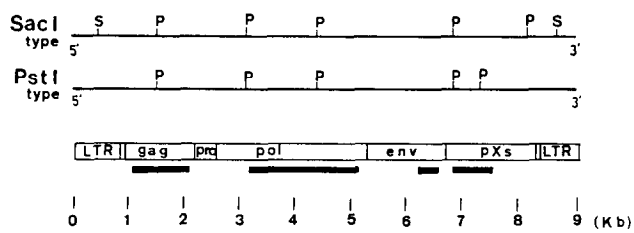

Fig. 3. Restriction endonuclease maps of two major subtypes of HTLV-1 in Japan. P: *Pst*I; S: *Sac*I. The thick bars under the genome represent regions used as probes as described in Materials and Methods.

Table 1. Distribution of two subtypes of HTLV-1 in ATL and HAM in Japan

Subtypes	HAM	ATL	Healthy carriers of HTLV-1[a]
*Pst*I type	8	9	4
*Sac*I type	7[b]	3	0
Defective type	0	5	0

[a]The cell lines 33 and 36 (corresponding to lanes 33 and 36 respectively in Figs. 1 and 2) were established from the same HTLV-1 healthy carrier; and cell line 33 is IL-2 independent whereas cell line 36 is IL-2 dependent. The cell lines 34 and 37 (lane 34 and 37 respectively in Figs. 1 and 2) were also established from the same HTLV-1 healthy carrier; cell line 34 is IL-2 independent whereas cell line 37 is IL-2 dependent. Thus the total number of healthy carrier individuals is four.
[b]Cell line 4 is classified as *Sac*I type (see text).

over those areas in Japan, indicating that genomic variations among HTLV-1 may not relate to their different geographical sources. It is worthwhile to note that the restriction enzyme map of an African isolate termed HTLV-1b is similar to a Japanese HTLV-1 isolated from cell line MT-2(9). The *Sac*I cuts in the LTR of HTLV-1b, suggesting that this African isolate may belong to the *Sac*I type of HTLV-1. One of large endemic areas of HTLV-1 could be Africa (14) and both ATL (15) and HTLV-1 associated spastic paraparesis (16) in Africa were also reported, although there has not yet been sufficent information available for the distribution of HTLV-1 genomic variations. Therefore it is interesting to test whether two subtypes of HTLV-1 in Japan could exist also in Africa. Analysis of other HTLV-1 isolates from Africa should further clarify this point.

Acknowledgments

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References

1. Poiesz B.J., Ruscetti F.W., Gazdar A.F., Bunn P.A., Minna J.D. & Gallo R.C., Proc Natl Acad Sci USA *77*, 7415-7419, 1980.
2. Hinuma Y., Nagata K., Hanaoka M., Nakai M., Matsumoto T., Kinoshita K., Shirakawa S. & Miyoshi I., Proc Natl Acad Sci USA *78*, 6476-6480, 1981.
3. Hatanaka M., Hematol Oncol *4*, 3-11, 1986.
4. Konishi H., Kobayashi N. & Hatanaka M., Mol Biol Med *2*, 273-283, 1984.
5. Gessain A., Barin F., Vernant J.C., Gout O., Maurs L., Calendar A. & deThe G., Lancet ii, 407-409, 1985.
6. Osame M., Usuku K., Izumo S., Ijichi N., Amitani H., Igata A., Matsumoto M. & Tara M., Lancet i, 1031-1032, 1986.
7. Yoshida M., Osame M., Usuku K., Matsumoto M. & Igata, A., Lancet i, 1085-1086, 1987.

8. Seiki M., Hattori S., Hirayama Y. & Yoshida M., Proc Natl Acad Sci USA *80*, 3618-3622, 1983.
9. Ratner L., Josephs S.F., Starcichi B., Hahn B., Shaw G.M., Gallo R.C. & Wong-Staal F., J Virol *54*, 781-790, 1985.
10. Mori K., Sabe H., Siomi H., Iino T., Tanaka A., Takeuchi K., Hirayoshi K. & Hatanaka M., J Gen Virol *68*, 499-506, 1987.
11. Sugamura K., Fujii M., Kannagi M., Sakitani M., Takeuchi K. & Hinuma Y., Int J Cancer *34*, 221-228, 1984.
12. Maeda M., Shimizu A., Ikuta K., Okamoto H., Kashihara M., Uchiyama T., Honjo T. & Yodoi J., J Exp Med 162, 2169-2174, 1985
13. Kobayashi N., Konishi H., Sabe H., Shigesasa K., Noma T., Honjo T. & Hatanaka M., EMBO J 3, 1339-1343, 1984.
14. Hunsmann G., Schneider J., Schmitt H. & Yamamoto N., Int J Cancer *32*, 329-332, 1983.
15. Hann B.H., Shaw G. H., Popovic M., LoMonico A., Gallo R. C. & Wong-Staal F., Int J Cancer *34*, 613-618, 1984.
16. Gessain A., Francis H., Sonan T., Giordano C., Akani F., Piquemal M., Caudie C., Malone G., Essex M. and deThe G., Lancet ii. 698, 1986.