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# Detection of Provirus in an HTLV-II Producer CD8+ T Cell Line by Polymerase Chain Reaction Combined with Digoxigenin-ELISA Method

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**A human T-cell leukemia virus type II (HTLV-II) producer cell line, designated HTLV-IIA, was established by cocultivation with leukocytes from an anti-human T-cell leukemia type I (HTLV-I) antibody-positive white male intravenous drug abuser and a healthy Japanese female. The cell line was examined for viral antigens by the indirect immunofluorescence method. The cytoplasm of over 80% of the cells was brilliantly stained. Cytogenetically, the cell line has a normal female karyotype. Electron microscopy of the HTLV-IIA disclosed many C-type retrovirus particles of mature, immature and non-cored types in the extracellular spaces. The surface markers of the transformed cells are CD2+, CD3+, CD4− and CD8+. To distinguish between HTLV-I and HTLV-II infection in the cell line, a method for detection of the HTLV-II provirus was developed by combining the polymerase chain reaction method with digoxigenin-enzyme-linked immunosorbent assay method.**

Key words: HTLV-II — CD8+ T-cell line — Polymerase chain reaction — Dig-ELISA

Human T-cell leukemia virus type I (HTLV-I) and human T-cell leukemia virus type II (HTLV-II) have been associated with adult T-cell leukemia (ATL),[1] HTLV-I-associated myelopathy[2] or tropical spastic paraparesis[3] and a T-cell variant of hairy-cell leukemia.[4] HTLV-I has been found to be highly endemic among patients and healthy carriers in the regions of Japan,[1] the Caribbean,[5] Africa[6] and the southeastern part of the United States.[7] On the other hand, only two patients have been found to have HTLV-II infection in unusual T cell malignancies resembling hairy-cell leukemia.[4, 8] The low number of patients with HTLV-II infection has made it difficult to determine its association with or causative role in any specific disease. Recently, a high rate of HTLV-II infection was found in seropositive intravenous (iv) drug abusers in New Orleans.[9] Serologically, however, it has not been possible to distinguish between HTLV-I and HTLV-II because of their relatively high amino acid sequence homology, resulting in substantial cross-reactivity.[10] But, recently, to distinguish between HTLV-I- and HTLV-II-infected persons, the polymerase chain reaction (PCR) method was developed for detection of the HTLV-II proviral genome.[11] This method and modifications of it involve radioactive labeling of the 5′ end of one oligonucleotide of the primer pair or probe.

We established an HTLV-II producer cell line to biologically characterize viral infected cells and we developed a method for non-radioactive detection of the HTLV-II proviral genome by combining the PCR method with digoxigenin(Dig)-ezyme-linked immunosorbent assay (ELISA) method.

A blood sample was obtained from a 35-year-old white male iv drug abuser in New York. The serum titer value of the anti-HTLV-I antibody was $\times 1024$ as measured by the particle agglutination test (PA) method (Fujirebio Inc.).[12] The sera reacted negatively to anti-human immunodeficiency virus type I (HIV-I) antibody and hepatitis surface antigen. Leukocytes from 20 ml of peripheral blood of the drug abuser were separated on Ficoll-Conray gradients (donor cells), after which they were cultured at $1\times10^6$/ml in a 35 mm Petri dish with RPMI 1640 medium, supplemented with 10% human cord serum, 10% fetal calf serum and antibiotics. Simultaneously, leukocytes ($1\times10^6$/ml) separated by Ficoll-Conray gradients from an anti-HTLV-I antibody-negative healthy Japanese female (recipient cells) were added to the dish containing the donor cells. Cultured cells were incubated at 37°C in a humidified atmosphere (5% $CO_2$) and fed twice weekly. After 3 to 4 weeks of co-culturing, scattered foci of cell aggregates were noted on macrophage sheets. These cell aggregates increased in size and number and the first subculture was made one month later. To date (January 1990), the transformed cells have been maintained in continuous culture for over 8 weeks. These cells are morphologically lymphoid and in suspension (Fig. 1). However, they seem to have grown more slowly than ones cultured by the co-culture method

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of anti-HTLV-I-positive cells and negative cells previously reported.[13] The transformed cells have the normal karyotype of recipient cells, i.e., 46,XX. The surface markers of the transformed cell line are CD2+ (92%), CD3+ (55%), CD4− (6%) and CD8+ (66%). The transformed cells were examined for viral antigens by indirect immunofluorescence using monoclonal antibody against HTLV-I-related antigens, i.e., P28 and P19. The cytoplasm of over 80% of the cells was brilliantly stained. Electron microscopy of the cells revealed many C-type retrovirus particles of mature, immature and non-cored types in the extracellular spaces (Fig. 2). To detect the HTLV-II provirus genome in both primary cells and transformed cells, DNA from these cells was subjected to PCR analysis with the following modification. DNA was prepared from samples of approximately $10^6$ cells of each type by SDS-proteinase K treatment, followed by phenol and chloroform extraction. Then 1 $\mu$g of DNA was subjected to 30 cycles of PCR amplification. A primer pair complementary to a conserved region of *pol* (SK58/SK59) was used in the amplification of HTLV-II DNA sequences.[14] The designated primer SK58 is complementary to the viral minus strand and SK 59 to the plus strand. SK58 is situated at the position from 4198 to 4217 in the *pol* region of HTLV-II virus sequences and SK59 from 4281 to 4300. After amplification, 10 $\mu$l of the reaction mixture was used for further analysis by 1.8% agarose gel electrophoresis, followed by denaturing in 0.5

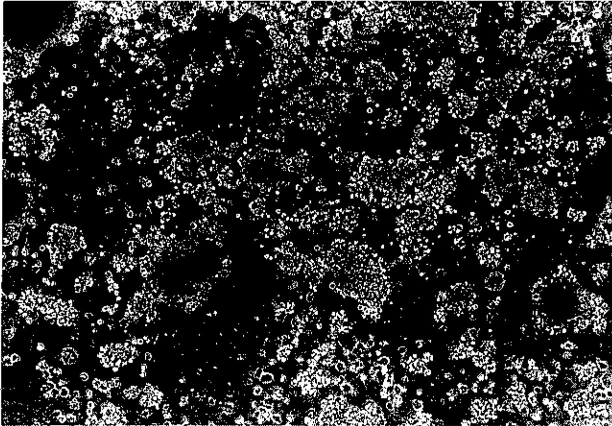

Fig. 1. Phase-contrast micrograph of the transformed HTLV-IIA cell line.

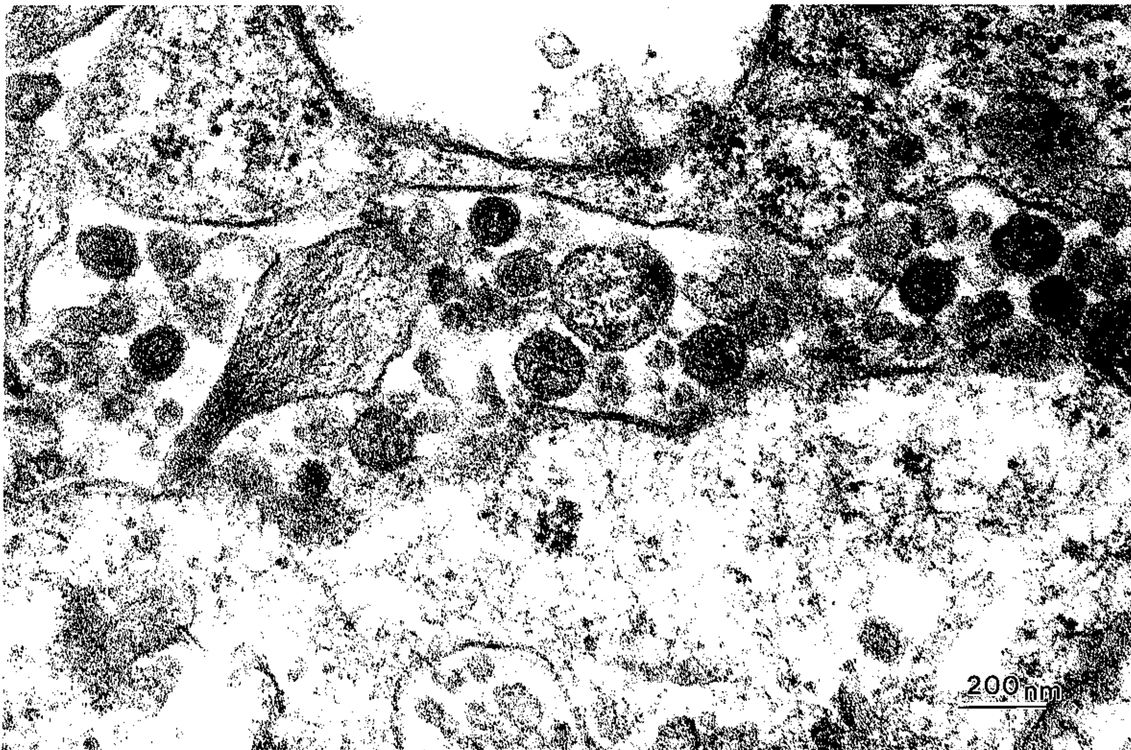


Fig. 2. Electron micrograph of the transformed HTLV-IIA cells showing extracellular type C retrovirus particles. ×57,000.

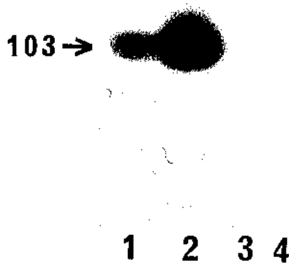


Fig. 3. Detection of HTLV-II provirus in the transformed HTLV-IIA cell line by PCR combined with Dig-ELISA method. Lane 1: primary cells. Lane 2: transformed cell line. Lane 3: healthy anti-HTLV-I antibody-negative cells. Lane 4: MT-2 cell line.

*M* NaOH/1.5 *M* NaCl solutions and transfer onto a 'Hybond N' (Amersham) nylon membrane. After prehybridization and hybridization with the 3′ end-Dig-11-dUTP-labeled SK60 probe, the filter was washed, blocked with blocking reagent, reacted with polyclonal sheep anti-Dig Fab fragments conjugated to alkaline phosphatase and colored with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate solutions. The SK60 probe was an oligonucleotide of a *pol* region in the HTLV-II provirus DNA sequences.[14] The designated SK60 probe is complementary to the viral plus strand. The SK60 is situated at the position from 4237 to 4276 in the *pol* region of HTLV-II virus genome sequences. Dig-11-dUTP labeling of the 3′-OH end SK60 probe was carried out with a DNA tailing kit (Boehringer Mannheim Inc.). The position of the 103-nucleotide (nt) HTLV-II-specific amplified DNA product was determined in both primary cells and transformed cells (Fig. 3). However, negative control cells (healthy anti-HTLV-I antibody-negative cells and the MT-2 cell line) were not detected.

A unique HTLV-II, isolated from a patient with a T-cell variant of hairy-cell leukemia, has been shown to be distinct from HTLV-I by immunologic[4] and molecular criteria.[15] The HTLV-II-infected T-cell line Mo-T was lethally X-irradiated and cocultivated with normal human peripheral blood lymphocytes. This cocultivation resulted in the transformation of normal cells.[16] In the present study, we also examined transformation by cocultivation with normal human peripheral blood lymphocytes and ones from the drug abuser. The transformed cells showed the T suppressor-cell phenotype (66%) and much small cell debris in scattered foci of cell aggregates with increased culture times. Recently, Rosenblatt *et al.*[17] reported integration of the HTLV-II genome in CD8+ T-cells from a patient with atypical hairy cell leukemia. Therefore, HTLV-II may be associated with CD8+ cell phenotype. Our HTLV-II producer cell line should contribute to further elucidation of the mechanisms involved in the pathogenesis of multiple maladies associated with HTLV-II. The molecular methods for HTLV-I, HTLV-II and HIV detection should be the most rigorous in asymptomatic individuals because of the low proportion of infected cells. Recently, a modification of the PCR method was used to detect HTLV-I, HTLV-II and HIV infection directly in DNA obtained from fresh blood samples.[9, 14, 18] The PCR method and its modifications are sensitive and rapid. However, hybridization probes of the PCR method are commonly labeled with the radioisotope $^{32}$P. Radioisotope probes are generally inconvenient to use and have short half lives. Recently, non-radioactive probes have been developed to overcome these deficiencies of radioisotope probes.[19] The present method for detection of the HTLV-II provirus genome, which combines the PCR method with Dig-ELISA method, might be suitable for distinguishing HTLV-II- from HTLV-I-infected cells or for the detection of various virus-infected cells by means of non-radioisotope analysis.

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REFERENCES

1) Hinuma, Y., Nagata, K., Hanaoka, M., Nakai, M., Matsumoto, T., Kinoshita, K., Shirakawa, S. and Miyoshi, I. Adult T-cell leukemia: antigen in an ATL cell line and detection of antibodies to the antigen in human sera. *Proc. Natl. Acad. Sci. USA*, **78**, 6476–6480 (1981).

2) Osame, M., Usuku, K., Izumo, S., Ijichi, N., Amitani, H., Igata, A., Matsumoto, M. and Tara, M. HTLV-I-associated myelopathy, a new clinical entity. *Lancet*, **i**, 1031–1032 (1986).

3) Gessain, A., Barin, F., Vernant, J. C., Gout, O., Maurs, L., Calender, A. and de Thé, G. Antibodies to human T-lymphotropic virus type-I in patients with tropical spastic

paraparesis. *Lancet,* **ii,** 407–409 (1985).
4) Kalyanaraman, V. S., Sarngadharan, M. G., Robert-Guroff, M., Miyoshi, I., Blayney, D., Golde, D. and Gallo, R. C. A new subtype of human T-cell leukemia virus (HTLV-II) associated with a T-cell variant of hairy cell leukemia. *Science,* **218,** 571–573 (1982).
5) Blattner, W. A., Kalyanaraman, V. S., Robert-Guroff, M., Lister, T. A., Galton, D. A. G., Sarin, P. S., Crawford, M. H., Catovsky, D., Greaves, M. and Gallo, R. C. The human type-C retrovirus, HTLV, in blacks from the Caribbean region, and relationship to adult T-cell leukemia/lymphoma. *Int. J. Cancer,* **30,** 257–264 (1982).
6) Saxinger, W., Blattner, W. A., Levine, P. H., Clark, J., Biggar, R., Hoh, M., Moghissi, J., Jacobs, P., Wilson, L., Jacobson, R., Crookes, R., Strong, M., Ansari, A. A., Dean, A. G., Nkrumah, F. K., Mourali, N. and Gallo, R. C. Human T-cell leukemia virus (HTLV-I) antibodies in Africa. *Science,* **225,** 1473–1476 (1984).
7) Blayney, D. W., Jaffe, E. S., Blattner, W. A., Cossman, J., Robert-Guroff, M., Longo, D. L., Bunn, P. A. and Gallo, R. C. The human T-cell leukemia/lymphoma virus associated with American adult T-cell leukemia/lymphoma. *Blood,* **62,** 401–405 (1983).
8) Rosenblatt, J. D., Golde, D. W., Wachsman, W., Giorgi, J., Jacobs, A., Schmidt, G. M., Quan, S., Gasson, J. C. and Chen, I. S. Y. A second isolate of HTLV-II associated with atypical hairy-cell leukemia. *N. Engl. J. Med.,* **315,** 372–377 (1986).
9) Lee, H., Swanson, P., Shorty, V. S., Zack, J. A., Rosenblatt, J. D. and Chen, I. S. Y. High rate of HTLV-II infection in seropositive IV drug abusers in New Orleans. *Science,* **244,** 471–475 (1989).
10) Shimotohno, K., Takahashi, Y., Shimizu, N., Gojobori, T., Golde, D. W., Chen, I. S. Y., Miwa, M. and Sugimura, T. Complete nucleotide sequence of an infectious clone of human T-cell leukemia virus type II: an open reading frame for the protease gene. *Proc. Natl. Acad. Sci. USA,* **82,** 3101–3105 (1985).
11) Ehrlich, G. D., Glaser, J. B., Lavigne, K., Quan, D., Mildvan, D., Sninsky, J. J., Kwok, S., Papsidero, L. and Poiesz, B. J. Prevalence of human T-cell leukemia/lymphoma virus (HTLV) type II infection among high-risk individuals: type-specific identification of HTLVs by polymerase chain reaction. *Blood,* **74,** 1658–1664 (1989).
12) Ikeda, M., Fujino, R., Matsui, T., Yoshida, T., Komoda, H. and Imai, J. A new agglutination test for serum antibodies to adult T-cell leukemia virus. *Gann,* **75,** 845–848 (1984).
13) Miyamoto, K., Tomita, N., Ishii, A., Nishizaki, T., Kitajima, K., Tanaka, T., Nakamura, T., Watanabe, S. and Oda, T. Transformation of ATLA-negative leukocytes by blood components from anti-ATLA-positive donors *in vitro. Int. J. Cancer,* **33,** 721–725 (1984).
14) Kwok, S., Ehrlich, G., Poiesz, B., Kalish, R. and Sninsky, J. J. Enzymatic amplification of HTLV-I viral sequences from peripheral blood mononuclear cells and infected tissues. *Blood,* **72,** 1117–1123 (1988).
15) Chen, I. S. Y., McLaughlin, J., Gasson, J. C., Clark, S. C. and Golde, D. W. Molecular characterization of genome of a novel human T-cell leukaemia virus. *Nature,* **305,** 502–505 (1983).
16) Chen, I. S. Y., Quan, S. G. and Golde, D. W. Human T-cell leukemia virus type II transforms normal human lymphocytes. *Proc. Natl. Acad. Sci. USA,* **80,** 7006–7009 (1983).
17) Rosenblatt, J. D., Giorgi, J. V., Golde, D. W., Ezra, J. B., Wu, A., Winberg, C. D., Glaspy, J., Wachsman, W. and Chen, I. S. Y. Integrated human T-cell leukemia virus II genome in CD8+ T cells from a patient with "atypical" hairy cell leukemia: evidence for distinct T and B cell lymphoproliferative disorders. *Blood,* **71,** 363–369 (1988).
18) Laure, F., Courgnaud, V., Rouzioux, C., Blanche, S., Veber, F., Burgard, M., Jacomet, C., Griscelli, G. and Brechot, C. Detection of HIV1 DNA in infants and children by means of the polymerase chain reaction. *Lancet,* **ii,** 538–540 (1988).
19) Forster, A. C., McInnes, J. L., Skingle, D. C. and Symons, R. H. Non-radioactive hybridization probes prepared by the chemical labelling of DNA and RNA with a novel reagent, photobiotin. *Nucleic Acids Res.,* **13,** 745–761 (1985).