Acta Medica Okayama

Volume 43, *Issue* 3 1989 *Article* 2

JUNE 1989

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Tadaatsu Akagi[*] Hiroshi Takata[†] Tadashi Yoshino[‡]
Norihiro Teramoto[**] Shoki Yano[††] Takashi Oka[‡‡]

[*]Okayama University,
[†]Okayama University,
[‡]Okayama University,
[**]Okayama University,
[††]Kochi Medical School,
[‡‡]Kochi Medical School,

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Tadaatsu Akagi, Hiroshi Takata, Tadashi Yoshino, Norihiro Teramoto, Shoki Yano, and Takashi Oka

Abstract

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KEYWORDS: human T-cell leukemia virus, rat T cell, immortalization

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*PMID: 2788356 [PubMed - indexed for MEDLINE]

Acta Med Okayama 43 (3) 143-151 (1989)

Immortalization of Rat Spleen and Thymus T Cells by Human T-Cell Leukemia Virus Type I

Tadaatsu Akagi*, Hiroshi Takata, Tadashi Yoshino, Norihiro Teramoto, Shoki Yano[a] and Takashi Oka[b]

Second Department of Pathology, Okayama University Medical School, Okayama 700, [a]Third Department of Internal Medicine and [b]Second Department of Pathology, Kochi Medical School, Kochi 781-51, Japan

Co-cultivation of thymus and spleen cells of Fisher and Lewis rats with lethally irradiated MT-2 cells harboring human T-cell leukemia virus type I (HTLV-I) resulted in the establishment of lymphoid cell lines, FIRT-1, FIRS-1, LERT-1, and LERS-1, respectively. Cells of these cell lines had rat T-cell characters as demonstrated by the positive reaction to monoclonal antibodies (MAbs) to rat T cell antigens (Thy 1 and pan T). They lacked surface immunoglobulins and strongly expressed rat interleukin-2 receptor antigen (Tac) and Ia antigen. Karyotypic analysis revealed that they had the normal rat karyotype in early cultures, but showed marked aneuploidy after long cultivation. None of them expressed HTLV *gag* proteins (p19 and p24) or virus particles, but they contained HTLV-I proviral DNA monoclonally and weakly expressed pX gene products ($p40^x$). They were not transplantable into syngeneic newborn rats.

Key words: human T-cell leukemia virus, rat T cell, immortalization

Human T-cell leukemia virus type I (HTLV-I) (1) is thought to be a causative agent of adult T-cell leukemia (ATL) (2, 3). However, only some infected people, mostly beyond middle age, develop overt disease (4). HTLV-I has been shown to easily immortalize human T cells *in vitro* when T cells were co-cultivated with HTLV-producing cells (5-7). These immortalized lymphocytes had the normal karyotype, although ATL cells had several inconsistent, but rather frequent karyotypic abnormalities (8, 9). These results strongly suggest that HTLV-I is needed for the initiation of ATL, but other factor(s) may participate in the complete carcinogenesis, implying multistep carcinogenesis of ATL. One of the predicted factors is the immunological response of hosts. For the elucidation of the host immune responses participating in pathogenesis of ATL, an experimental model of inbred animals may be a useful tool. In the present paper, the authors report the successful immortalization of T cells from inbred strains of rats.

*To whom correspondence should be addressed.

Materials and Methods

Animals. Inbred Fisher and Lewis rats were purchased from Charles River Japan Inc. and bred in our laboratory.

Cells. MT-2 cells are HTLV-I-producing human cord blood leukocytes established by co-cultivation with ATL cells (10). Thymuses and spleens were aseptically removed from young-adult female rats, minced with scissors, and squeezed between two glass slides. Liberated cells were passed through a 150-mesh platinum screen. Spleen cells were suspended in 0.85% NH_4Cl solution to lyse red blood cells. After several washings in phosphate buffered saline (PBS), the cells were suspended in culture medium. All cells were cultivated in RPMI 1640 medium supplemented with 15% fetal calf serum (FCS).

Co-cultivation. After adherent cells were removed by incubating spleen and thymus cells in Petri dishes at 37°C for 30 min, the cells were suspended in culture medium and placed in 35-mm Petri dishes at a density of 5×10^6/ml.

After 2 days, 1×10^6 lethally irradiated(10,000 R) MT-2 cells were added to each dish. Control cultures were rat spleen cells, rat thymus cells and irradiated MT-2 cells, which were not co-cultured. All cultures were maintained in RPMI 1640 supplemented with 15% FCS at 37°C in a humidified 5% CO_2 atmosphere. Cultures were fed twice a week. Rat purified interleukin-2 (IL-2) (Inter-Cell Technologies, Inc., Somerville, NJ, USA) was added to cultures of thymus cells of Lewis rats at a concentration of 10%. IL-2 was not added to any of the other cultures.

Antisera. Mouse monoclonal antibodies (MAbs) to surface markers of rat lymphoid cells and rat immunoglobulin (Ig) M were purchased from Serotec Inc. The specificities of the MAbs are listed in Table 1. MAbs to HTLV-I p19 and p24 *gag* proteins were purchased from Sera-lab Inc. A MAb to pX gene products, NCC-pX-1G, which was supplied by S. Watanabe (Epidemiology Division, National Cancer Center Research Institute), was produced against a fused protein of a part of pX protein and a bovine growth hormone synthesized in *Escherichia coli* (11).

Immunofluorescence (IF) analysis. Surface markers of lymphoid cells were examined by membrane IF test. Unfixed cells (5×10^5/0.1 ml) were first incubated with 0.1 ml of mouse MAbs to rat T-cell antigens, major histocompatibility antigen complex, class II antigens (Ia antigens), rat IgM, and rat IL-2 receptor (Tac antigen) at a 1 : 200 or a 1 : 1,000 dilution. After washing with PBS, the cells were mixed with 0.1 ml of fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG goat $F(ab')_2$ (Tago, Burlingame, California) at a 1 : 20 dilution and incubated at 37°C for 1 h. For detection of HTLV-I core antigens, smeared cells fixed in acetone for 10 min were first treated with antisera to HTLV-I p19 or p24 *gag* proteins at

Table 1 Specificities of monoclonal antibodies against rat lymphocyte-related antigens

Name	Specificities	Class of immunoglobulin
MRC OX-7	Anti rat and mouse Thy 1-1	IgG1
MRC OX-8	Anti rat supressor/cytotoxic T cells majority of NK cells and thymocytes (homologous to human OKT-3 and mouse Lyt2)	IgG1
MRC OX-17	Anti α chain of rat Ia antigen (homologous to mouse I-E)	IgG1
MRC OX-19	Anti whole T cells and thymocytes (homologous to human OKT-1, Leu 1 and mouse Lyt1)	IgG1
W3/25	Anti rat helper/inducer T cells (homologous to human OKT-4 and mouse L3T4)	IgG1
MARM-4	Anti rat mu-4 (IgM)	IgG1
MRC OX-39	Anti IL-2 receptor (Tac antigen) on activated rat T cells	IgG1

a 1 : 80 dilution and then with FITC conjugated anti-mouse IgG reagent.

Light and electron microscopy. The cells were observed under a phase-contrast microscope. Smeared and fixed cells were stained with Giemsa.

Pelleted cells were fixed in 3% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in graded ethanol, and embedded in an epoxy resin. Ultra-thin sections were stained with uranyl acetate and lead citrate and then examined under a Hitachi H-500 electron microscope.

Chromosome analysis. Chromosome banding was carried out by the ordinary G-banding technique (12), and the banding patterns were analyzed according to the standard rat karyotype proposed by the Committee for a Standardized Karyotype of *Rattvs norvegicus* (13).

Southern blot hybridization. The assay was performed as described previously (14). Cellular DNA was digested with an excess of EcoRI (Takara Shuzo, Kyoto), which did not cut the HTLV-I provirus genome internally, and hybridized with probe DNA, pHT-1 (M) 3.9, containing *LTR*, *pX*, *env*, and a part of *pol* of the HTLV-I genome (obtained from Dr. K. Shimotohno, National Cancer Center Research Institute).

Transplantation test. FIRT-1, FIRS-1, LERT-1, or LERS-1 cells were injected intraperitoneally into syngeneic newborn rats at a dose of 1×10^6 cells.

Results

Establishment of cell lines. About one month after co-cultivation, proliferation of lymphoid cells became evident. These cells formed aggregates on adherent fibroblastic and macrophage-like cells. The first subculture was done about 1-2 months after co-cultivation. Thereafter, the cells were consistently subcultured. The establised cell lines were designated FIRS-1, FIRT-1, LERS-1, and LERT-1 according to their origin: spleen cells of Fisher rats, thymus cells of Fisher rats, spleen cells of Lewis rats and thymus cells of Lewis rats, respectively. These cells proliferated in sus-

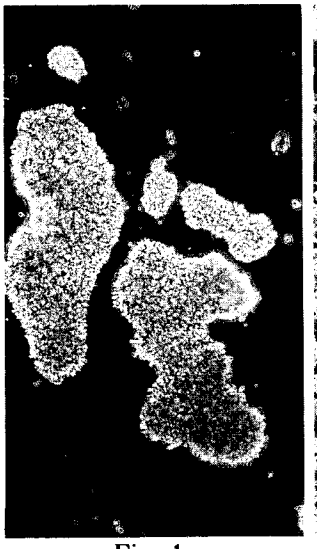

Fig. 1

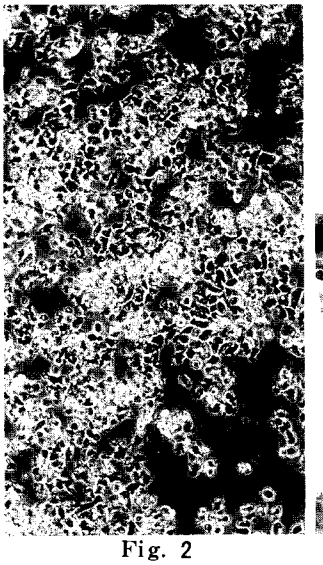

Fig. 2

Fig. 3

Fig. 1 Phase contrast micrograph of LERT-1 cells. Note the large cell clumps floating in suspension. ×50.

Fig. 2 Phase contrast micrograph of LERS-1 cells. The cells proliferate adhering to the culture vessels or forming small cell clusters. ×100.

Fig. 3 Giemsa staining of smeared LERT-1 cells. The cells have round or irregularly shaped nuclei, distinct nucleoli, and basophilic cytoplasm with perinuclear halos. ×400.

pension without addition of IL-2 and were subcultured at a 1:5 split ratio every 3 days. The growth of LERT-1 cells was IL-2-dependent in the early passages. The cells from the LERT-1 line mainly formed floating cell clumps (Fig. 1). On the other hand, FIRS-1, FIRT-1, and LERS-1 cells proliferated mainly in single cell suspension with inconspicuous cell clumps and had a tendency to adhere to the surface of culture vessels (Fig. 2).

Control rat spleen and thymus cells and irradiated MT-2 cells cultured alone degenerated within 2 months.

Light and electron microscopy. The cells from each cell line were irregularly outlined, small or medium-sized lymphoid cells, having scanty or abundant cytoplasm with prominent perinuclear halo and round or oval nuclei with distinct nucleoli (Fig. 3). The adherent cells had short cytoplasmic processes. Ultrastructurally, most nuclei had smooth nuclear envelopes and distinct nucleoli. However, the nuclei of FIRT-1 cells showed irregular indentation and invagination of nuclear envelopes. The cytoplasm had abundant free ribosomes and polysomes, mitochondria in variable numbers and sizes, and prominent Golgi complexes, especially in LERT-1 cells (Figs. 4, 5). Cell debris were frequently seen, but no virus particles were observed.

Cell characterization. Immunocytological characters demonstrated by specific antibodies to various surface markers are presented in Table 2. All cell lines were positive for pan T-cell markers (MRC OX-7, MRC OX-19), but the strength of reactivities and the percentage of positive cells were variable. Nearly 30-50% of LERT-1

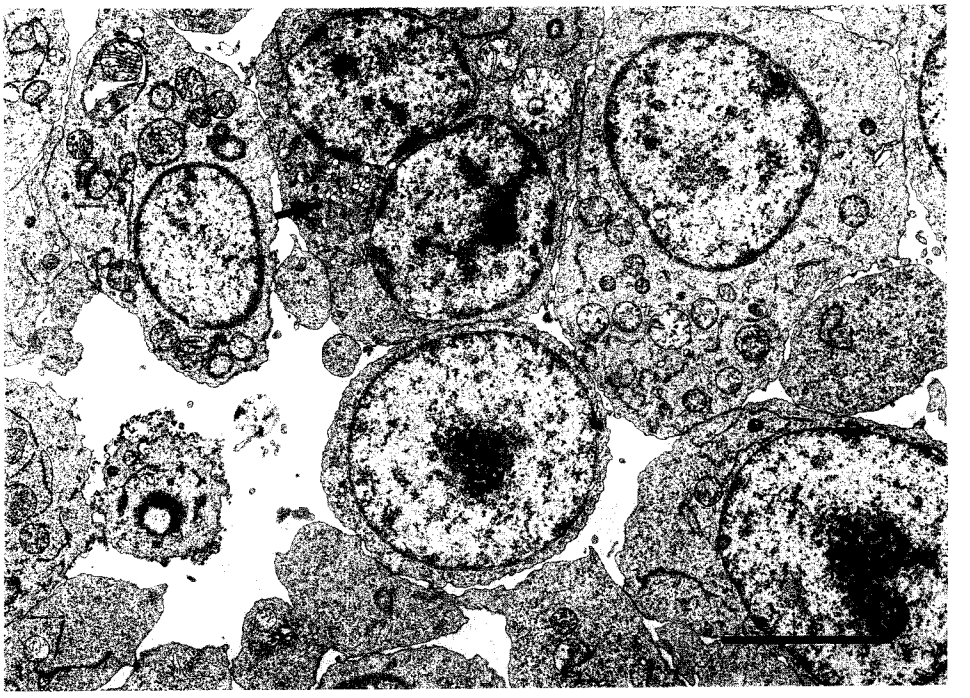

Fig. 4 Electron micrograph of FIRS-1 cells. The cells have round nuclei with smooth nuclear envelopes and distinct nucleoli, and have moderate numbers of mitochondria of variable size. Sometimes, binucleated cells are seen (arrow). No virus particles are seen. Bar= 5 μm.

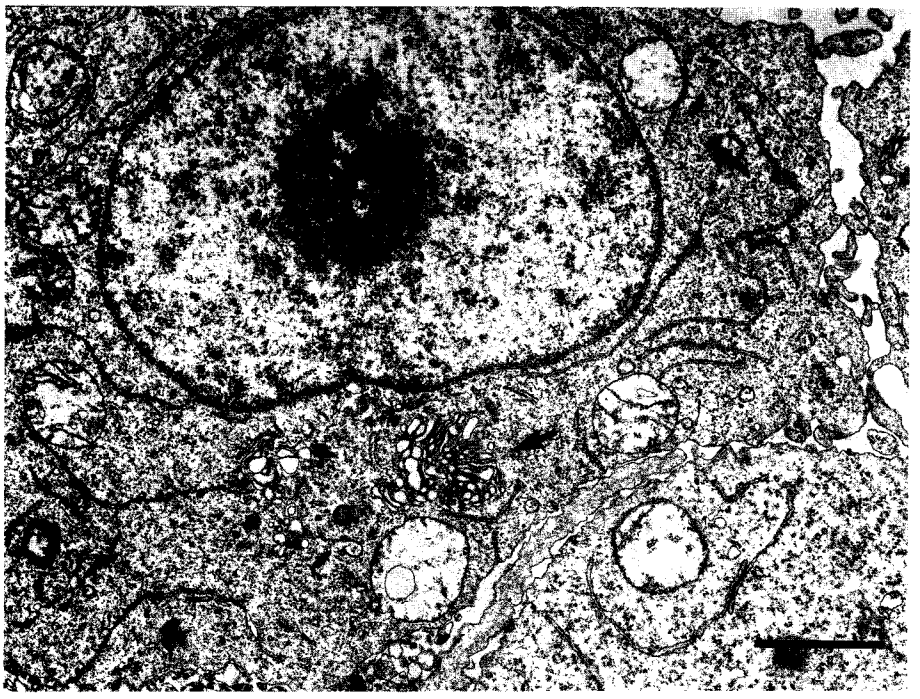

Fig. 5 Electron micrograph of LERT-1 cells. The cells have somewhat uneven nuclear envelopes and distinct nucleoli. The cytoplasm has abundant free ribosomes and polysomes, and well developed Golgi complex (arrow). No virus particles are seen. Bar= 2 μm.

Table 2 Reactivities of the rat lymphoid cell lines to monoclonal antibodies against rat lymphocyte- and HTLV-I-related antigens by immunofluorescence

Antisera	LERS-1	LERT-1	FIRS-1	FIRT-1
MRC OX-7 (Thy 1)[a]	+[b] (5)[c]	+ (30-50)	+ (<1)	+ (<1)
MRC OX-8 (T8)	−	−	−	−
MRC OX-17 (Ia)	++ (20)	++ (20)	++ (20)	++ (20)
MRC OX-19 (T1)	+ (100)	++ (100)	+ (100)	++ (100)
W3/25 (T4)	+ (50-60)	−	−	−
MARM-4 (IgM)	−	−	−	−
MRC OX-39 (Tac)	+++ (100)	+++ (100)	+++ (100)	+++ (100)
Anti pX	+ (<1)	+ (10)	+ (50)	+ (20-30)
Anti p19	−	−	−	−
Anti p24	−	−	−	−

a: Antigens recognized by the antisera.
b: Intensity of immunofluorescence of antigen-positive cells.
c: Percentage of antigen-positive cells.

cells, but only less than 5% of the other cells, were weakly positive for Thy 1-1 antigen. MRC OX-19 antibody for detecting whole T cell and thymocyte antigens reacted weakly or moderately to about 100% of cells from all the cell lines (Fig. 6).

Reactivity against suppressor/cytotoxic T-cell antigens (MRC OX-8) was demonstrated in none of the 4 cell lines, and helper/inducer T-cell antigens (W3/25) were

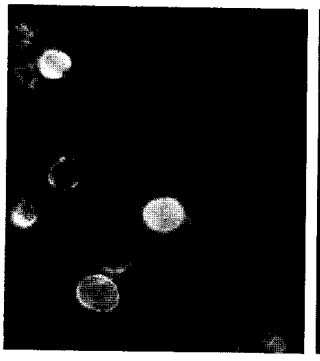

Fig. 6

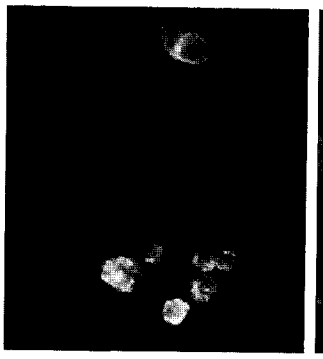

Fig. 7

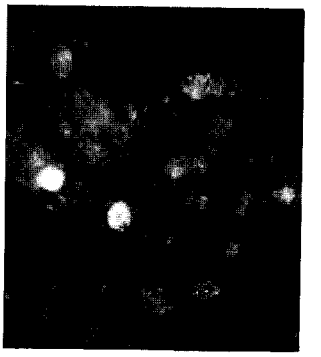

Fig. 9

Fig. 6 T1 antigens of FIRT-1 cells. Almost 100% of the cells show positive membrane immunofluorescence. ×400.

Fig. 7 IL-2 receptor (Tac) antigen of FIRS-1 cells. All the cells show brilliant membrane fluorescence. ×400.

Fig. 9 pX gene products ($p40^x$) of FIRS-1 cells detected by immunofluorescence. About half of the cells show a positive reaction. ×400.

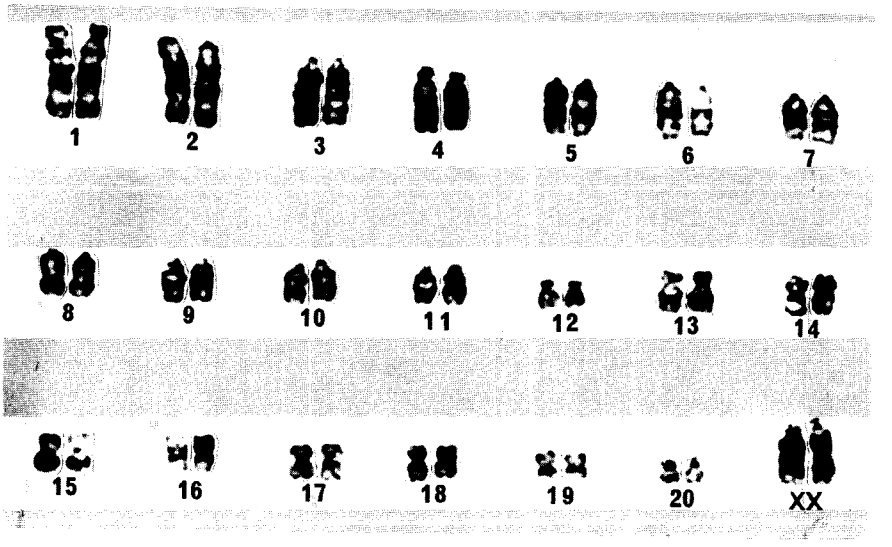

Fig. 8 Karyotypic analysis of FIRT-1 cells by G-banding.

detected only in LERS-1 cells. Nearly 100% of cells from all the cell lines were strongly positive for rat IL-2 receptor antigen (MRC OX-39), but none were positive for human IL-2 receptor antigen (Fig. 7). Class II major histocompatibility complex antigens and rat Ia antigens (MRC OX-17) were detected in all the cell lines, but surface IgM was detected in none.

Karyotypic analysis revealed that all the cell lines had the normal rat karyotype in early cultures (Fig. 8). After long cultivation, however, marked aneuploidy appeared.

HTLV core antigens and pX gene products. Almost 100% of FIRS-1 cells, 50% of LERT-1 and FIRT-1 cells, and only 1% of LERS-1 cells reacted with MAbs to pX gene products by the ABC method. The IF test also demonstrated positive reactions, but in smaller numbers of cells (Table 2,

Fig. 9). Positive reactions occurred both in the nuclei and cytoplasm.

None of the cell lines reacted with MAbs to HTLV *gag* proteins, p19 or p24, even after induction by addition of 5-iodo-2'-deoxyuridine (50 μg/ml) for 4 days.

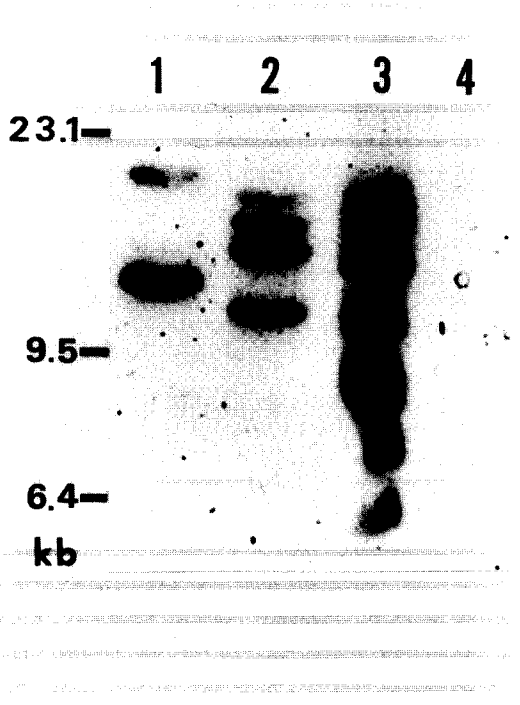

Fig. 10 Detection of HTLV-I provirus genome in the rat cell lines. EcoRI digests of cellular DNA were analyzed by Southern blot hybridization. Lane 1, LERT-1; lane 2, FIRS-1; lane 3, MT-2; lane 4, normal rat thymocytes.

Southern blot hybridization. DNA of FIRS-1 and LERT-1 cells digested with EcoRI was hybridized with an HTLV probe DNA. FIRS-1 and LERT-1 DNAs gave 4 and 2 hybridizable bands, respectively (Fig. 10). FIRT-1 and LERS-1 cells were lost in an accident and were not available for examination.

Transplantability. None of the syngeneic newborn rats transplanted intraperitoneally with cells of any of the cell lines produced tumors.

Discussion

HTLV-I immortalizes not only human T cells (5-7), but also lymphocytes of animals such as the monkey (15), rabbit (16), cat (17), hamster (18), and rat (19). Tateno *et al.* (19) established 3 HTLV-I-producing lymphoid cell lines from Wistar-King-Aptekman rats. In this study, we established 4 T-cell lines of Fisher and Lewis rats, FIRT-1, FIRS-1, LERT-1, and LERS-1, by co-cultivation with HTLV-I-producing human T-cells. These cell lines had the normal rat karyotype in early culture passages and did not express the human T-cell antigens or human IL-2 receptor antigen (data not shown), revealing that they were derived from rat lymphocytes, and not from co-cultured MT-2 cells. They were also shown to originate from rat T cells by the presence of rat Thy 1 and pan T antigens and the absence of surface IgM. They strongly expressed rat, and not human, IL-2 receptor antigen. Abnormal karyotypes that appeared in the later passages may be the nonspecific secondary alterations frequently observed after long culture. FIRS-1 and LERT-1 cells had integrated HTLV-I proviral DNA as demonstrated by Southern blot hybridization, but did not produce HTLV-I virions and core antigens, and are analogous to hamster cell lines in their characters (18). HTLV-I does not have a usual oncogene. However, the putative transforming gene, pX, has recently received attention because the pX gene product ($p40^x$) has been implicated in the initiation and maintenance of cell transformation by its transacting transcriptional activation of the LTR of HTLV-I (20-23). In this context, it is interesting that the pX gene product ($p40^x$) was detected immunohistochemically in all these rat cell lines. These

animal non-producer cell lines apparently mimic human ATL cells which do not produce HTLV-I virions *in vivo*, but express them after short cultivation *in vitro* (24). If the cells of rat cell lines have full genomes of HTLV-I, but can not replicate them, these cell lines may provide useful tools for the elucidation of the regulatory mechanism participating in the expression of HTLV-I virions in ATL cells. Alternatively, they might have been immortalized by the integration of defective viruses. Further studies are required to clarify these points.

We did not succeed in transplanting the cells into syngeneic newborn rats. On the contrary, the rat cell lines immortalized in a similar way by Tateno *et al.* (19) produced HTLV-I virions and were transplantable. This discrepancy might be partly due to the difference in animal strains and transformation procedures although more sophisticated analysis is required in order to make a comparison. As to the lack of transplantability some possibilities can be considered. The immortalized cells might acquire or express new antigens acting as transplantation resistance antigens, or the immortalization might not necessarily mean the transformation, but rather an intermediate step on the way to the final carcinogenesis as suggested by the multistep theory of carcinogenesis.

Acknowledgments. The authors thank Mr. T. Zohta for the photographs, Miss K. Urakami for chromosome analysis, Miss M. Shiotani for cell culture, and Miss M. Kobayashi for preparation of the manuscript. This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan (No.62570162).

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Received February 18, 1989; accepted March 7, 1989