Jpn. J. Cancer Res. 85, 808–818, August 1994

HTLV-II Non-integrated Malignant Lymphoma Induction in Japanese White Rabbits Following Intravenous Inoculation of HTLV-II-infected Simian Leukocyte Cell Line (Si-IIA)

Kazuhiko Hayashi,[1,6] Nobuya Ohara,[1] Tirtha Raj Koirala,[1] Hideo Ino,[1] Hong Li Chen,[1] Norihiro Teramoto,[1] Eisaku Kondo,[1] Tadashi Yoshino,[1] Kiyoshi Takahashi,[1] Masao Yamada,[2] Noriko Tomita,[3] Kanji Miyamoto,[4] Koji Fujimoto,[5] Yasuhiro Yoshikawa[5] and Tadaatsu Akagi[1]

[1]Second Department of Pathology, [2]Department of Virology, Okayama University Medical School, 2-5-1 Shikata-cho, Okayama 700, [3]Okayama Red Cross Blood Center, 3-36 Izumi-cho, Okayama 700, [4]School of Health Science, Okayama University, 2-5-1 Shikata-cho, Okayama 700 and [5]Tsukuba Primate Center for Medical Science, National Institute of Health, 1 Hachimandai, Tsukuba-shi, Ibaraki 305

Lymphoma induction in rabbits by an unknown factor derived from an HTLV-II-producing simian (Cynomolgus) leukocyte cell line (Si-IIA) is reported. Thirteen of 17 male Japanese white rabbits (76%) inoculated intravenously with Si-IIA cells developed malignant lymphoma including Hodgkin-like lymphoma between 62 and 167 days after inoculation. Histologically, there was extensive diffuse or nodular infiltration of either large cell type or mixed type lymphoma cells in many organs, frequently involving the spleen, liver, lymph nodes and kidneys, and less frequently the thymus, bone marrow, lungs, heart, skin and gastrointestinal tract. Hodgkin-like lymphoma was also observed in two rabbits. Chromosomal analysis of five cell lines established from tumor-bearing rabbits revealed the male rabbit karyotype. The immunophenotype of these tumor cells was usually T-cell ($CD5^{+\text{ or }-}$, $RT1^{+}$, $RT2^{+\text{ or }-}$, $CD45^{+}$, $CD4^{-}$, $RABELA^{-}$ and MHC class $II\text{-}DQ^{+}$) except for Hodgkin-like lymphoma cells which expressed only CD45. However, integration of the HTLV-II provirus genome could not be demonstrated in the tumor tissues or any of the rabbit cell lines by polymerase chain reaction or Southern blot analysis. Moreover, no lymphoma was induced by inoculation of HTLV-IIC, MOT (other HTLV-II-producing human cell lines) or TALL-1 (control). Two of four rabbits injected with cell-free pellets from Si-IIA cultures died of malignant lymphoma (15–20 days). Five irradiated rabbit cell lines were inoculated but only one (Ra-SLN) induced lymphoma in 1 of 3 rabbits at 27 days. Neither *Herpesvirus saimiri* nor *Herpesvirus ateles* (simian oncogenic viruses) was detected in Si-IIA cells by immunofluorescence testing. These data suggest that the high rate of lymphoma induction in rabbits may be caused not by only HTLV-II or well known simian oncogenic viruses, but rather by an unknown passenger agent derived from Si-IIA or HTLV-IIA, with which Si-IIA was established.

Key words: Malignant lymphoma — Rabbit — Simian leukocyte — HTLV-II

Human T cell lymphotropic virus type II (HTLV-II) was initially isolated from a patient with a T cell variant of hairy cell leukemia[1] and has subsequently been isolated only sporadically in malignancy[2] or in non-malignant maladies.[3] Recently, a high rate of HTLV-II infection has been noted among intravenous drug users[4] and in patients with acquired immunodeficiency syndrome (AIDS),[5] HTLV-I associated myelopathy (HAM)/tropical spastic paraparesis (TSP)[6] and in some geographic populations.[7] HTLV-II infection has also been detected in a patient with large granular lymphocyte leukemia[8] and in one of nine patients with mycosis fungoides.[9] However, the etiological role of HTLV-II remains obscure.

We previously established an HTLV-II-producing $CD8^{+}$ T cell line (HTLV-IIA) by cocultivating leukocytes from a 35-year-old white male intravenous drug abuser with those from a healthy Japanese woman.[10] We also established an interleukin-2 (IL-2) dependent HTLV-II-producing $CD4^{+}$ T cell line (HTLV-IIC) by cocultivating leukocytes from a 54-year-old black male with those from a healthy Japanese woman.[11] A simian (Cynomolgus monkey) leukocyte cell line, Si-IIA, was established by cocultivation with HTLV-IIA cells,[12] and Si-IIA again immortalized human T cells.[13,14] Furthermore, HTLV-II-specific polyclonal and monoclonal antibodies were developed against a synthetic oligopeptide of an HTLV-II envelope protein.[15,16]

In this study, we induced malignant lymphomas in Japanese white rabbits by intravenous inoculation of

[6] To whom correspondence and reprint requests should be sent.

HTLV-II-producing simian leukocyte cell line (Si-IIA) cells, and obtained results indicating that the induction may not be directly associated with HTLV-II infection.

MATERIALS AND METHODS

Inoculation of cells and pellets from culture supernatants Specific-pathogen-free male Japanese white rabbits (2–3 kg in weight, obtained from Japan LAMB, Hiroshima), in which spontaneous malignant lymphoma development has never been observed, to our knowledge, were inoculated intravenously with 1.0×10^6 to 1.0×10^8 cells or the pellet from 400–1100 ml of supernatant (Table I). HTLV-II-producing simian (Si-IIA), or human (HTLV-IIC and MOT[1]) leukocyte cell lines and an HTLV-unifected cell line (TALL-1) were all lethally irradiated (100 Gy, γ irradiation) before inoculation, and the effectiveness of irradiation was verified by failure of the cells to grow in culture. Culture supernatants obtained from Si-IIA culture (5×10^5 cells/ml) were first centrifuged at 8,000*g* for 30 min to remove cell debris (Hitachi Himac CR20, Tokyo) and then at 100,000*g* for 60 min to obtain the pellets (Hitachi Himac Centrifuge SCP85H).

Immunization of rabbits with an HTLV-II-specific oligopeptide Seven rabbits were immunized subcutaneously with complete Freund's adjuvant and a KLH-conjugated synthetic oligopeptide (1 mg at the first, and 500 μg at the second and subsequent immunizations) corresponding to the amino acids 171–196 of the HTLV-II envelope protein, which is a specific region for HTLV-II.[15] Immunization was performed four or seven times at 2-week intervals.

Establishment of tumor cells in culture Establishment of tumor cell lines was tried from eight tumors (4 rabbits) and peripheral blood leukocytes (PBL) (1 tumor-bearing rabbit) and from normal-appearing spleens and PBL from 3 non-tumor-bearing rabbits as controls. The cells from minced tumor tissues or the normal-looking spleens were allowed to grow in plastic tissue flasks with RPMI-1640 medium supplemented with 10% fetal calf serum and antibiotics and cultured at 37°C in a humidified atmosphere (5% CO_2). Half of the medium was replenished every 3–4 days.

Morphology Tumor-bearing rabbits appearing ill were killed with intravenous injection of pentobarbital sodium (Abbott Laboratories, USA). All animals were killed by the end of 7 months. Samples from heart, lungs, spleen, liver, kidneys, thymus, lymph nodes, bone marrow (vertebra, sternum and femur), skin, muscles and gastrointestinal tract were examined histologically for the presence of microscopic tumors in all of the rabbits studied. Sections of formalin-fixed paraffin-embedded tumor tissues were stained with hematoxylin-eosin and observed by light microscopy. The cultured cells were observed by phase-contrast microscopy. For electron microscopy, the tissues or cultured cells were fixed in phosphate-buffered 3% glutaraldehyde overnight at 4°C, post-fixed in 1% osmium tetroxide for 1 h at 4°C, dehydrated in a series of ethanol rinses, and embedded in an epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and then examined with an electron microscope (JEM 1200 EX-II, JEOL, Tokyo).

Phenotypic analysis Paraffin-embedded tissue sections or cytocentrifuged smears were immunostained by the avidin-biotin-peroxidase complex (ABC) method (immuno mark biotin avidin universal kit; ICN Biochemical, USA) or the peroxidase antiperoxidase (PAP) method using antibodies to rabbit CD45, CD5, CD4, MHC class II DQ (Serotec, Oxford, England), RT1, RT 2 (antibodies to rabbit T cells, Cedarlane Laboratories, Canada) or RABELA (rabbit bursal equivalent lymphocyte antisera, Cedarlane Laboratories). Tumor specific antigen (T antigen) was examined immunohistochemically in acetone-fixed rabbit lymphoma cell line cells using sera from tumor-bearing rabbits as a primary antibody and the ABC method.

Chromosomal analysis Chromosomal analysis of the cultured cells was performed by the Q-banding technique.[17]

Detection of anti-HTLV-II antibodies Sera were examined by means of the particle agglutination test (SERODIA-HTLV-I, Fujirebio, Tokyo), which can also detect anti-HTLV-II antibodies because of antigenic cross-reactivity due to the high amino acid sequence homology between HTLV-I and HTLV-II.

PCR and Southern blot analysis To detect the HTLV-II provirus genome in tumor tissues, cultured cells or peripheral blood leukocytes (Experiment 1–3 in Table I), DNA was analyzed by polymerase chain reaction (PCR), combined with a digoxigenin enzyme-linked immunosorbent assay (ELISA) method.[10] HTLV-IIA or Si-IIA cells were used as the positive control, and peripheral leukocytes of a healthy adult human or rabbit were used as the negative control. Extracted DNA was subjected to 30 cycles of PCR amplification using the following primer pairs: SK58 and SK59 (pol of HTLV-II), SK110 and SK111 (pol of HTLV-II) (Vienna Lab., Austria), TLV-127 and TLV-128 (env of HTLV-II) or TLV-131 and TLV-132 (tax of HTLV-II) (Genemed Biotechnologies, USA). The reaction mixture was size-fractionated by 1.2% agarose gel electrophoresis, transferred to a nylon membrane, and hybridized with DNA probes complementary to the HTLV-II DNA sequence[18]: SK60 (pol), SK188 (pol) (Vienna Lab.), TLV-217 (env), and TLV-219 (tax) (Genemed Biotechnologies), respectively. For Southern blot analysis, the DNAs were digested by *Eco*R1 or *Bam*HI and hybridized with a ^{32}P-labeled DNA probe complementary to a *Bam*HI-*Hin*dIII fragment (1.4 kbp) in a pX region

of HTLV-II (kindly donated by Dr. K. Shimotohno, National Cancer Center Research Institute, Tokyo).

Reverse transcriptase (RT) assay RT activity in the pellets obtained by ultracentrifugation (100,000*g*, 60 min) from the culture supernatants of five newly established rabbit cell lines was assayed with a non-radioactive reverse transcriptase assay ELISA kit (Boehringer-Mannheim, Germany). Supernatants from Si-IIA and MT-2 cultures were used as positive controls, and those from MOLT-4 and TALL-1 as negative controls.

Screening tests for simian oncogenic viruses in the Si-IIA cell line a) Immunofluorescence (IF) test: The IF test was used for detection of viral antigens of simian oncogenic viruses: simian T-cell leukemia virus (STLV-I), Epstein-Barr-related virus from a cynomolgus monkey (Cyno-EBV), *Herpesvirus saimiri* and *H. ateles*. Si-IIA cells were smeared on the slides, air-dried for over 30 min and fixed in acetone for 10 min at room temperature. The fixed cells were then incubated for 1 h at room temperature with simian antibody-positive sera against STLV-I, Cyno-EBV (Tsukuba Primate Center, Ibaraki), *H. saimiri* or *H. ateles* (Virus Reference Laboratory Inc., USA) and antibody-negative sera from a healthy monkey. After rinsing three times with phosphate-buffered saline (PBS), the slides were incubated with fluorescein isothionate (FITC)-labeled anti-monkey IgG (Cappel, USA) for 30 min. Then the slides were washed three times with PBS and examined with a fluorescence microscope.

Table I. Summary of Inoculations, Survival Times, and Pathologic Findings

Experiment	Rabbit	Inoculum	Survival after inoculation (days)	Pathologic findings
1	R-C	Si-IIA, 1×10^8	119	ML
	R-D	Si-IIA, 1×10^8	159 [k]	ML
	R-G	Si-IIA, 8×10^7	180 [k]	NSL
	R-H	Si-IIA, 1×10^7	202 [k]	NSL
	R-K	Si-IIA, 6×10^7	83 [k]	ML
	R-L	Si-IIA, 1×10^6	90	ML
	R-X	Si-IIA, 3×10^6	128	ML
	R-Y	Si-IIA, 3×10^6	111	ML
	R-g	Si-IIA, 3×10^6	62	ML
	R-h	Si-IIA, 3×10^6	167	ML
2	R-A	(Peptide+CFA)×7 — Si-IIA, 7×10^7	180 [k]	NSL
	R-B	(Peptide+CFA)×7 — Si-IIA, 7×10^7	200 [k]	NSL
	R-S	(Peptide+CFA)×4 — Si-IIA, 3×10^6	90	ML
	R-T	(Peptide+CFA)×4 — Si-IIA, 3×10^6	150 [k]	ML
	R-Z	(Peptide+CFA)×4 — Si-IIA, 3×10^6	69	ML
	R-a	(Peptide+CFA)×4 — Si-IIA, 3×10^6	70	ML
	R-b	(Peptide+CFA)×4 — Si-IIA, 3×10^6	172 [k]	ML
3	R-14	HTLV-IIC, 1×10^6	181 [k]	NSL
	R-15	HTLV-IIC, 1×10^6	177 [k]	NSL
	R-16	HTLV-IIC, 1×10^6	173 [k]	NSL
	R-19	HTLV-IIC, 5×10^6	168 [k]	NSL
	R-24	HTLV-IIC, 5×10^6	151 [k]	NSL
4	R-40	MOT, 1×10^7	122 [k]	NSL
	R-41	MOT, 1×10^7	122 [k]	NSL
	R-42	MOT, 1×10^7	122 [k]	NSL
	R-43	MOT, 2×10^7	121 [k]	NSL
	R-44	MOT, 5×10^7	121 [k]	NSL
5	R-I	TALL-1, 1×10^8	150 [k]	NSL
	R-J	TALL-1, 1×10^7	150 [k]	NSL
	R-c	TALL-1, 3×10^6	171 [k]	NSL
	R-d	TALL-1, 3×10^6	173 [k]	NSL
	R-e	TALL-1, 3×10^6	202 [k]	NSL
6	R-21	Pellets of supe (1100 ml) from Si-IIA	15	ML
	R-26	Pellets of supe (800 ml) from Si-IIA	20	ML
	R-28	Pellets of supe (600 ml) from Si-IIA	180 [k]	NSL
	R-29	Pellets of supe (400 ml) from Si-IIA	180 [k]	NSL

Abbreviations: k, killed; ML, malignant lymphoma; NSL, non-specific lesion; CFA, complete Freund's adjuvant; supe, supernatant.

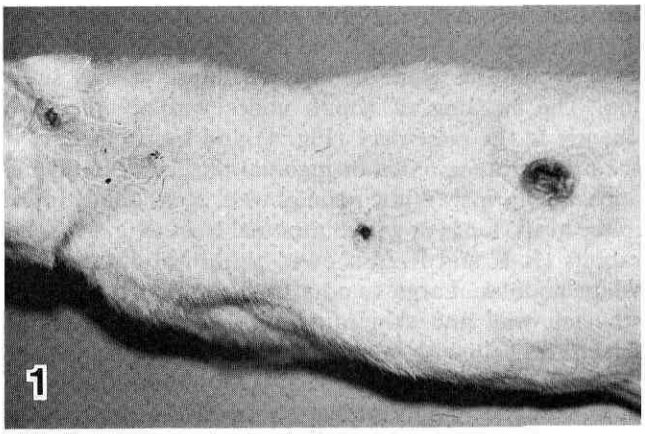
b) Detection of proviral DNA of STLV-I: DNA extracted from Si-IIA cells was examined for the presence of STLV-I-related sequences by Southern blot hybridization with HTLV-I probe, which can also detect STLV-I because of the 90% homology of DNA sequence between HTLV-I and STLV-I (Hayami, 1992). Cellular DNA was digested with *Eco*RI and hybridized with the ^{32}P-labeled DNA probe, pHT-1 (M) 3.9, containing LTR, pX, env, and a part of pol of the HTLV-I genome, which was kindly supplied by Dr. K. Shimotohno (National Cancer Center Research Institute, Tokyo).

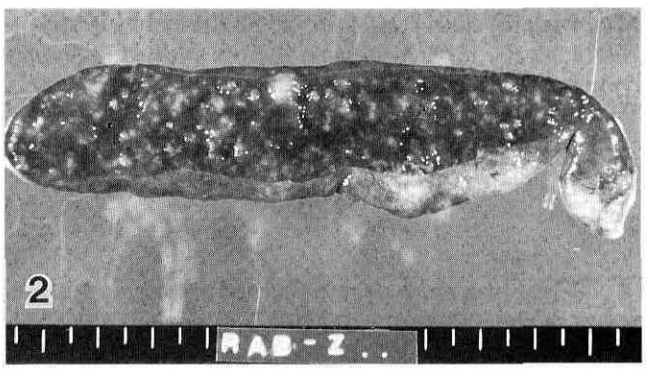
Tumorigenicity of established rabbit lymphoma cell lines in other normal rabbits Female rabbits (2–2.5 kg in weight) were inoculated intravenously with 1×10^7 irradiated (100 Gy) or non-irradiated cells of Ra-L-IIA, Ra-K, Ra-D2, Ra-SLN or Ra-Zsp (Table II).

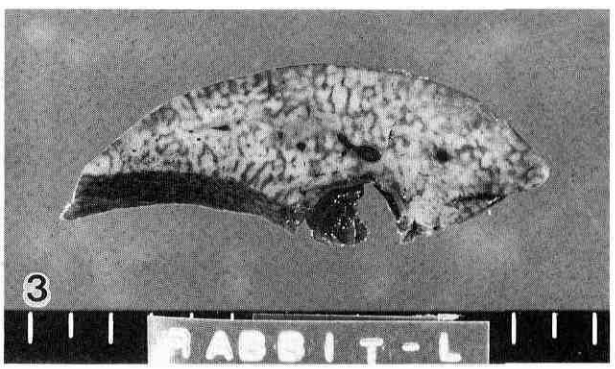
RESULTS

Tumor incidence in rabbits inoculated with HTLV-II-producing cells or cell-free pellets Tumor development in the rabbits examined is summarized in Table I. Of the ten rabbits inoculated with HTLV-II-producing simian Si-IIA cells, eight rabbits (80%) developed malignant lymphomas 62 to 167 days after inoculation (Experiment 1). This tumor induction was not inhibited by immunization with the HTLV-II env oligopeptide (Experiment 2). In this experiment, tumor incidence was 71% (5/7). Malignant lymphoma was also induced in 2 of 4 rabbits (50%) by injection of the cell-free pellets obtained from Si-IIA culture supernatants after a short latent period (15–20 days) (Experiment 6). However, no tumor was detected in the rabbits inoculated with HTLV-II-producing human cells, HTLV-IIC and MOT or HTLV-II unrelated TALL-1 cells during 121–202 days after inoculation.

Macroscopic characteristics of Si-IIA-induced tumors Most of the rabbits injected with Si-IIA cells appeared physically normal, except for anorexia, until the day of death. Some developed dyspnea and became extremely ill and lethargic, and some appeared to make a recovery from illness. A few rabbits (R-K, R-T, R-Z) showed several skin tumors (Fig. 1). On necroscopy, most of the tumor-bearing rabbits revealed splenomegaly and/or







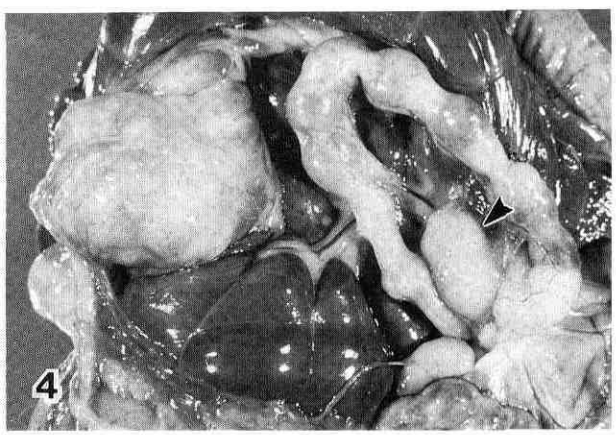


Fig. 1. Multiple skin tumors observed in R-K.

Fig. 2. Cross-section of enlarged spleen showing multiple small white nodules in R-Z.

Fig. 3. Cross-section of the liver showing band-like white lesions in R-L.

Fig. 4. Mesenteric lymph node swelling (arrowhead) and a huge tumor of the wall of the gastrointestinal tract in R-b.

hepatomegaly with multiple white nodules (about 1–5 mm in diameter) (Figs. 2 and 3). The cortex of kidneys showed multiple white nodules or diffuse whitish swelling. The swelling of lymph nodes was observed frequently in the mesentery (Fig. 4) and less frequently in the mediastinum, axilla or inguinal regions. The thymus appeared normal. White nodules were occasionally found in the heart, lungs or gastrointestinal tract (Fig. 4). Some rabbits (R-K and R-Z) showed peritoneal disseminated white nodules. Large tumors in the spleen, mesentery, stomach wall and skin often showed central necrosis, which was yellowish in color. Two of four rabbits inoculated with cell-free pellets of Si-IIA cultures also developed multiple tumors similar to the Si-IIA cell-induced tumors described above.

Microscopic characteristics of Si-IIA-induced tumors Histologically, Si-IIA-induced tumors showed malignant lymphoma with leukemic infiltration in many organs. Two histological types of malignant lymphomas were observed: 1) Non-Hodgkin's lymphoma, diffuse, large cell type or mixed type, which was predominant and frequently observed (Fig. 5a); 2) Hodgkin-like lymphoma, which was observed only in two rabbits (R-D and R-b) (Fig. 5b). All the spleens of tumor-bearing rabbits except one (R-T) showed severe or moderate infiltration lesions of lymphoma cells. In one case (R-D), Hodgkin-like cells with giant pleomorphic nuclei and prominent nucleoli were observed in one of the nodular lesions, while diffuse large lymphoma was demonstrated in the other nodular lesions. Regarding the lymph node infiltration, most lymph nodes (R-C, R-K, R-Y, R-h, R-T and R-a) showed partial or diffuse sinus infiltration of lymphoma cells, while large swollen mesenteric lymph nodes (R-L, R-S, R-Z and R-26) were destroyed by prominent infiltration of lymphoma cells. Secondary infiltration of lymphoma cells in the thymus was seen in one case (R-K). Focal bone marrow involvement of lymphoma cells was detected only in two cases (R-L, R-S). The livers in all but four cases (R-D, R-T, R-a and R-b) revealed severe periportal and mild sinusoidal infiltration of lymphoma cells (Fig. 6). Mild or moderate multiple tumor cell infiltration lesions with some diffuse nodules

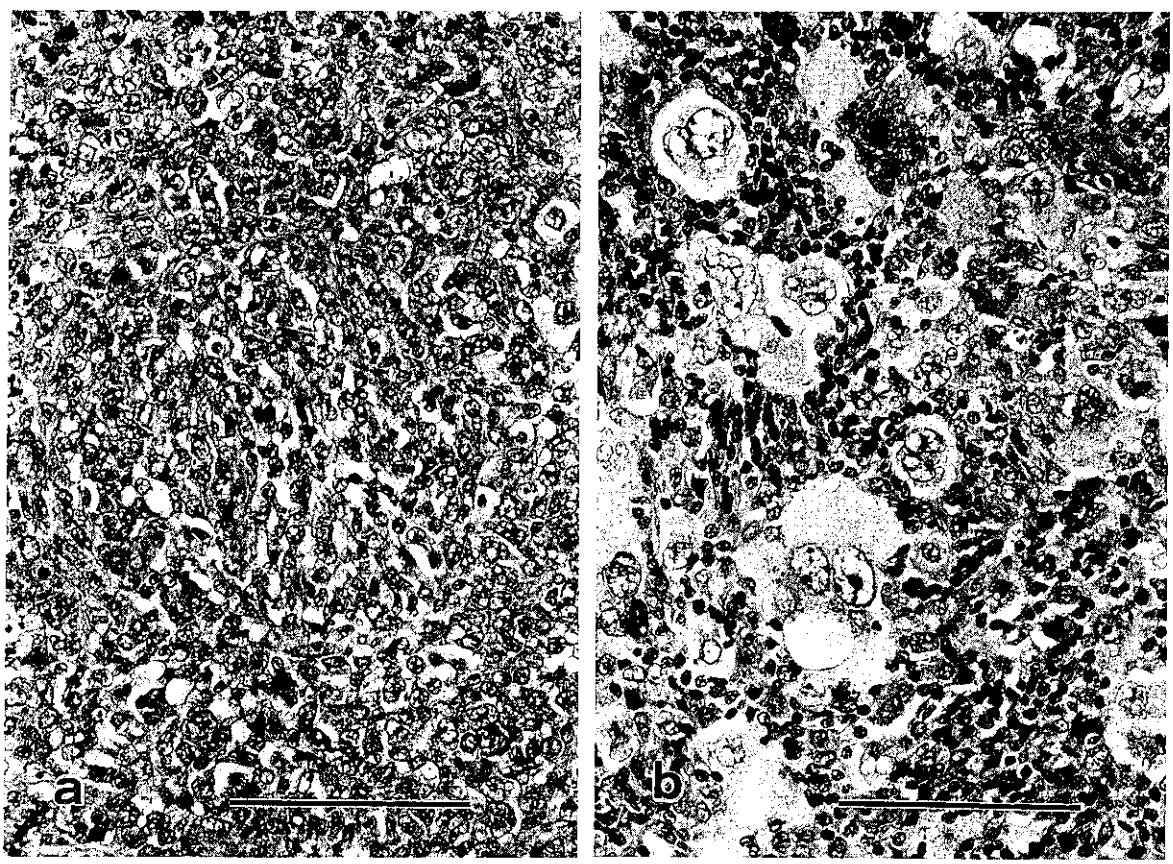


Fig. 5. (a) Diffusely proliferated large cell lymphoma in one of the nodules of the spleen (R-D). (b) Hodgkin-like cell infiltrated in another nodule of the same spleen (R-D). Bars=100 μm

were observed in the cortex of all kidneys. Tumor cells often proliferated in the glomeruli, which appeared as scattered multiple round nodules (R-D, R-K, R-Y, R-h, R-T and R-b) (Fig. 7). Mild or moderate perivascular infiltration lesions of tumor cells were often detected in the lungs (R-C, R-L, R-X, R-Y, R-g, R-h, R-S, R-Z, R-a and R-26), but rarely in the heart (R-K, R-T and R-a). The lungs frequently exhibited bronchopneumonia or pulmonary edema. Lymphoma cells also invaded the gastrointestinal tract (R-K and R-b), adrenal gland (R-K, R-L and R-T), peritoneum (R-K and R-Z), muscles (R-K, R-T and R-Z), and skin (R-K, R-T and R-Z) (Fig. 8). Atypical lymphocytes (leukemic lymphoma cells) were occasionally observed in the vessels. In case R-a, all tumor tissues exhibited complete necrosis with severe calcification.

Cytosmear examination of peripheral blood in rabbits injected with Si-IIA cells frequently revealed some atypical lymphocytes (2–4%) in more than half of the cases and increased atypical lymphocytes (10%) in one case with lymphocytosis (R-26). Some of the atypical lymphocytes had a flower-like nucleus (Fig. 9).

Cell lines established from the tumors or peripheral blood

The five cell lines were established only from the tumor-bearing rabbits, and designated Ra-L-IIA (from R-L), Ra-K (from R-K), Ra-D2 (from peripheral blood of R-D), Ra-Zsp (from R-Z), and Ra-SLN (from R-S). These cell line cells, which were lymphoid in morphology, have been maintained for more than 10 months without IL-2. Four cell lines (Ra-L-IIA, Ra-K, Ra-Zsp and Ra-SLN) grew rapidly (doubling time: 3–7 days) in suspension culture as aggregates or as single cells (Fig. 10), but Ra-D2 cells were extremely large and grew slowly (doubling time: more than 2 weeks) (Fig. 11). Ultrastructurally, the cell-line cells were characterized by an indented euchromatic nucleus and moderately differ-

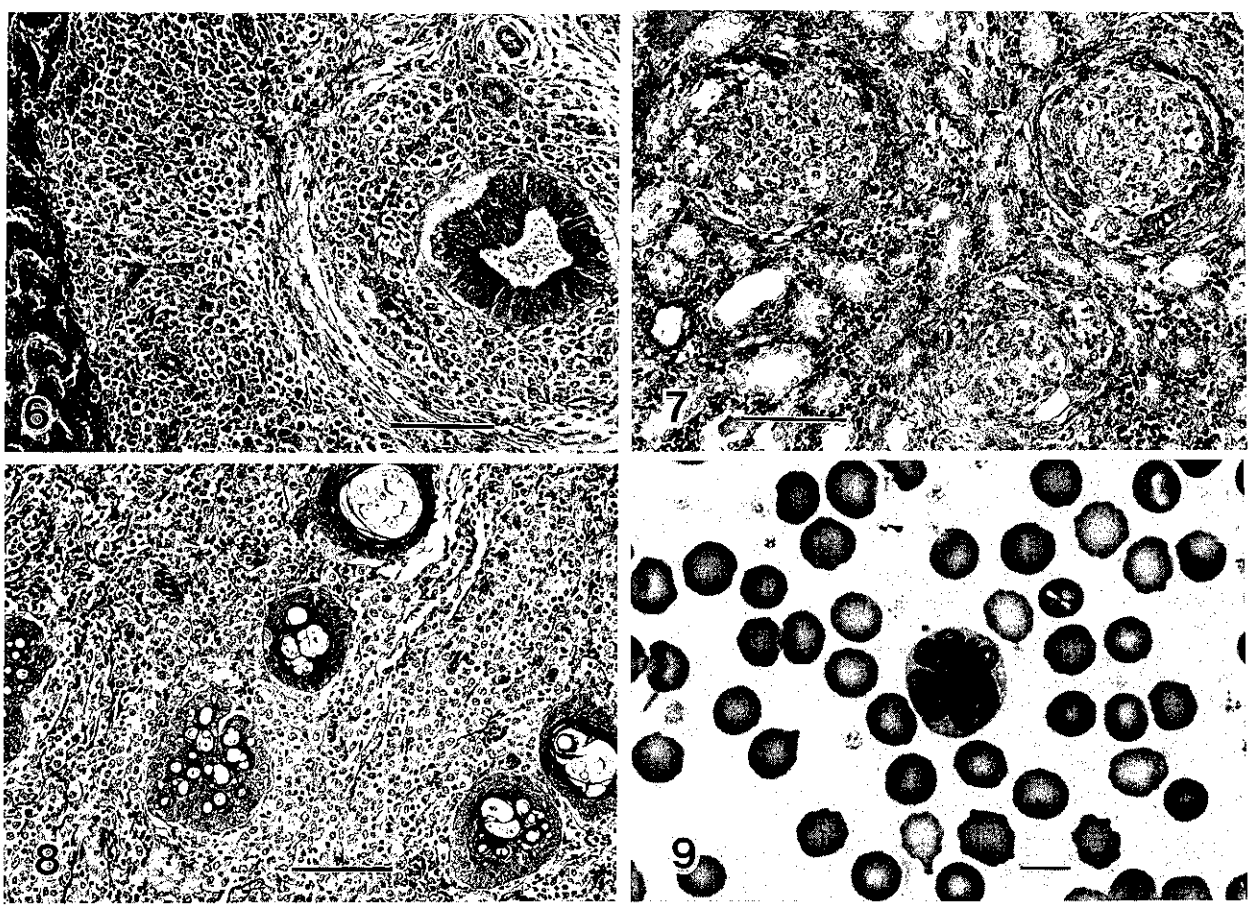
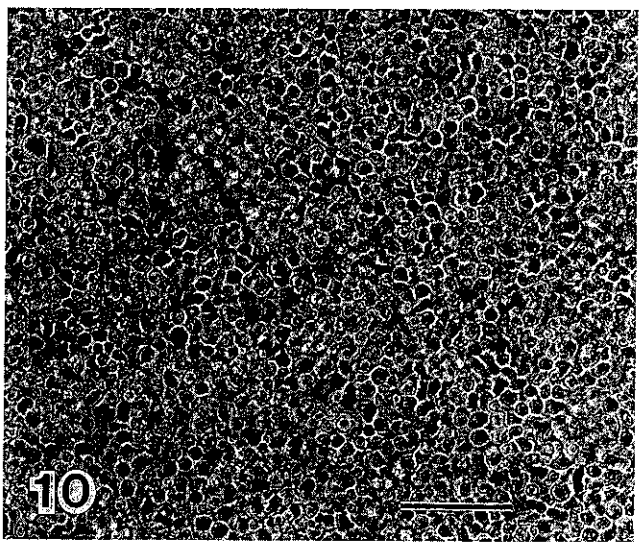


Fig. 6. The liver showing severe periportal infiltration of lymphoma cells (R-L). Bar=100 μm
Fig. 7. Some glomeruli with proliferation of lymphoma cells (R-b). Bar=100 μm
Fig. 8. Diffuse dermal infiltration of lymphoma cells in the skin (R-K). Bar=100 μm
Fig. 9. A flower-like atypical lymphoid cell observed in peripheral blood (R-L). Bar=10 μm

entiated cytoplasm, which contained numerous ribosomes, scattered mitochondria, strands of rough endoplastic reticulum, some multivesicular bodies, microtubules and glycogen deposits. Some cells also had microvilli. However, virus particles were not found.

Immunophenotype Immunohistochemically, tumor cells or cell line cells from R-K, R-L, R-S and R-Z were rabbit CD45$^+$, CD5$^{+\text{ or }-}$, MHC class II-DQ$^+$, RT1$^+$, RT2$^{+\text{ or }-}$, CD4$^-$ and RABELA$^-$ (Fig. 12). However, Hodgkin-like large tumor cells of R-D showed positive immunore-



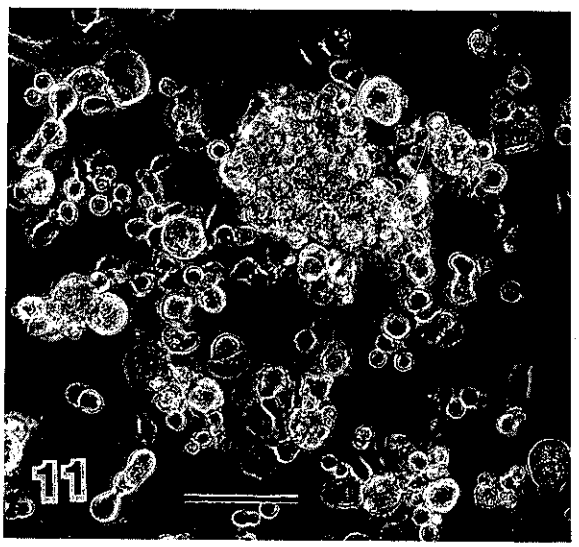


Fig. 10. Rapidly growing lymphoid cell line of Ra-K. Phase-contrast. Bar=100 μm
Fig. 11. Slowly growing huge lymphoid cell line of Ra-D2. Phase-contrast. Bar=100 μm

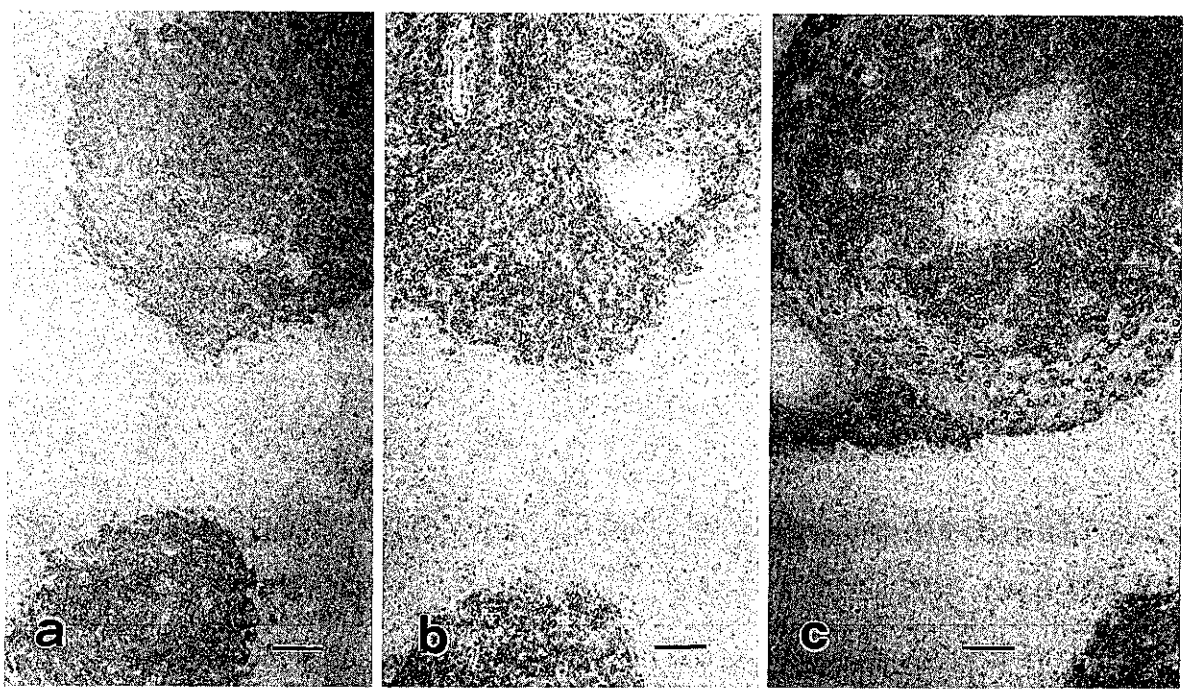


Fig. 12. Periportal infiltrated lymphoma cells (R-L) showing positive immunoreactivity for rabbit CD45 (a), CD5 (b) and MHC class II-DQ (c). Bars=100 μm

activity only for rabbit-CD45, and Ra-D2 cells derived from R-D were positive only for rabbit CD45 and RT1, and negative for rabbit CD4, CD5, MHC class II-DQ, RT2 and RABELA.

T antigen was not detected in any cell line by immunohistochemical methods using the sera obtained at autopsy from each of the tumor-bearing rabbits.

Chromosomal analysis All cell lines had the karyotype of male rabbits. Chromosomal abnormalities such as hypodiploidy were observed in Ra-D2 and Ra-K, while the other cell lines had a diploid karyotype.

Detection of HTLV-II infection The antibody titers against HTLV-I/II antigens, assayed by the particle agglutination method, were in the range of 32- to more than 128-fold in the sera from the rabbits inoculated with HTLV-II-producing cells (Experiment 1–4) but became negative in the sera at autopsy from some of the tumor-bearing rabbits (R-D, R-L, R-X). In the peripheral blood leukocytes obtained from most of the rabbits inoculated with HTLV-II-producing cell lines (Experiments 1 and 3), except for the rabbits immunized with HTLV-II-specific oligopeptides (Experiment 2) and some of Experiment 1 (R-C, R-D, R-X, R-Y) or Experiment 3 (R-15, R-19), the amplified DNA product of HTLV-II-positive genome was demonstrated at the 103 nucleotide position by PCR using the SK60 probe. In the negative control, tumor samples examined (R-C, R-D, R-K, R-L) and 5 rabbit tumor cell lines except the positive control, no amplified HTLV-II DNA was detected by PCR using 4 probes: SK60, SK188, TLV-217 and TLV-219. Southern blot analysis on these samples using *Eco*RI and the probe for the pX region of HTLV-II revealed the integration of HTLV-II provirus genome at the position of about 8 kbp only in DNA of the positive control (Si-IIA) but not in DNA of the established rabbit lymphoma cell lines or TALL-1 (Fig. 13). Southern blot analysis using another enzyme, *Bam*HI, also showed the presence of the provirus genome at the position of about 3.4 kbp only in the positive control.

RT assay of the supernatant from the rabbit cell lines No RT activity was detected in the samples from 5 rabbit lymphoma cell lines and negative controls, while positive RT activity was confirmed in the positive controls.

Detection of viral antigen of simian oncogenic viruses in Si-IIA Si-IIA cells showed positive reaction only with the sera from STLV-I-positive monkeys. No Si-IIA cells gave positive reaction with the antibody-positive sera against Cyno-EBV, *H. saimiri* or *H. ateles* or sera from an antibody-negative healthy monkey.

Detection of proviral DNA of STLV-I in Si-IIA The proviral sequence of STLV-I, which can be hybridized with HTLV-I probe, was not detected in DNA from Si-IIA by Southern blot analysis.

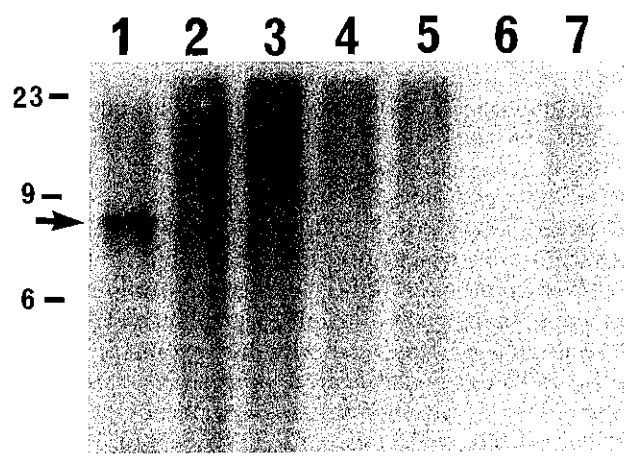


Fig. 13. Absence of proviral genome of HTLV-II in rabbit tumor cell lines by Southern blot analysis. Extracted DNA from rabbit cell lines (lanes 2–6; Ra-D2, Ra-K, Ra-L-IIA, Ra-SLN, Ra-Zsp, respectively), the positive control (Si-IIA) (lane 1) and the negative control (TALL-1) (lane 7) were digested with *Eco*RI and hybridized with ^{32}P-labeled probe (1.4 kbp) containing a pX region of HTLV-II. Only the positive control shows the presence of the proviral genome of HTLV-II at the position of about 8 kbp (arrow).

Table II. Tumorigenicity of Rabbit Tumor Cell Lines Established in Other Rabbits

Rabbit no.	Inoculum (1×10^7)	Survival (days)	Tumor (ML)
45	X-ray irradiated Ra-L-IIA	90 [k]	−
46	X-ray irradiated Ra-L-IIA	84 [k]	−
47	X-ray irradiated Ra-L-IIA	90 [k]	−
48	X-ray irradiated Ra-K	90 [k]	−
49	X-ray irradiated Ra-K	90 [k]	−
50	X-ray irradiated Ra-K	90 [k]	−
51	X-ray irradiated Ra-Zsp	69 [k]	−
52	X-ray irradiated Ra-Zsp	87 [k]	−
53	X-ray irradiated Ra-SLN	27	+
54	X-ray irradiated Ra-SLN	91 [k]	−
55	X-ray irradiated Ra-SLN	91 [k]	−
56	X-ray irradiated Ra-D2	91 [k]	−
57	X-ray irradiated Ra-D2	91 [k]	−
32	Non-irradiated Ra-L-IIA	6	+
33	Non-irradiated Ra-L-IIA	6	+
58	Non-irradiated Ra-L-IIA	91 [k]	−
11	Non-irradiated Ra-K	150	−
67	Non-irradiated Ra-K	90 [k]	−
70	Non-irradiated Ra-K	14 [k]	−
34	Non-irradiated Ra-Zsp	137 [k]	−
35	Non-irradiated Ra-Zsp	120 [k]	−
81	Non-irradiated Ra-SLN	23	+
82	Non-irradiated Ra-SLN	50 [k]	−
12	Non-irradiated Ra-D2	180 [k]	−
13	Non-irradiated Ra-D2	153 [k]	−

Abbreviations: k, killed; ML, malignant lymphoma.

Tumorigenicity of the newly established lymphoma cell lines in normal rabbits The results of this experiment are summarized in Table II. One (No. 53) of 13 rabbits inoculated with the irradiated cell lines, which were observed for 3 months, died of malignant lymphoma 27 days after the inoculation. Some of the rabbits inoculated with non-irradiated cell lines also died of malignant lymphoma in a short time (Nos. 32, 33 and 81).

DISCUSSION

HTLV-II has not been clearly linked to a particular disease in rabbits or humans, although HTLV-II can transform leukocytes of rabbits *in vitro*, and the HTLV-II provirus genome is integrated.[12] Infectious transmission of HTLV-II to rabbits *in vivo* was also demonstrated by PCR of the HTLV-II pol sequence; this occurred most frequently in peripheral blood mononuclear cells of rabbits (4/6) inoculated with MOT, and no evidence of disease was found in HTLV-II-inoculated rabbits observed for as long as 24 weeks.[19] In this study, infectious transmission of HTLV-II was also confirmed in most of the rabbits inoculated with Si-IIA or HTLV-IIC cells as demonstrated by PCR. This transmission was not observed in rabbits immunized with HTLV-II env-specific peptide, but malignant lymphomas frequently developed after a short latency period (62–172 days) in rabbits injected with Si-IIA cells, irrespective of immunization. Chromosomal analysis of 5 cell lines established from tumor-bearing rabbits revealed the rabbit karyotype, indicating that malignant lymphomas observed in the rabbits inoculated with Si-IIA cells resulted from the neoplastic transformation of the host lymphocytes. The development of malignant lymphoma 15–20 days after inoculation in two of four rabbits injected with cell-free pellets from Si-IIA cultures provides further evidence for the rabbit origin of induced tumors and suggests that malignant lymphomas of rabbits may be caused by a cell-free factor, possibly a kind of virus. However, no lymphoma was induced by inoculation of HTLV-IIC or MOT cells (other HTLV-II-producing human cell lines) or TALL-1 cells (control). The absence of tumors in the controls and the rabbits inoculated with HTLV-IIC or MOT excludes the possibility that these malignant lymphomas were induced by only HTLV-II or by an endogenous oncogenic virus activated by the inoculated leukocyte cultures. This speculation is further supported by the observation that no integration of HTLV-II provirus genome was detected in the tumor tissues or any of the cell lines established by PCR or Southern blot analysis. On the other hand, neither *H. saimiri* from the squirrel monkey nor *H. ateles* from the spider monkey was detected in Si-IIA cells derived from leukocytes of a cynomolgus monkey by IF test although it is well known that both *H. saimiri* and *H. ateles* can induce malignant lymphoma (small lymphocytic or lymphoblastic type) in about 20–100% of New Zealand white rabbits in a short time.[20] Cyno-EBV[21] could not be detected in Si-IIA cells by IF test. Proviral DNA of STLV-I in Si-IIA cells could not be demonstrated by the probe of HTLV-I pX, which had 90% homology with the nucleotide sequence of STLV-I pX.[22] Positive reactivity of HTLV-II-producing Si-IIA cells with sera from STLV-I positive-monkeys in the IF test could be explained by the fact that almost all the components of STLV-I are antigenetically cross-reactive with HTLV-I, which is serologically cross-reactive with HTLV-II.[15, 22] These results suggest that high rates of lymphoma induction may be caused by an unknown passenger virus derived from Si-IIA or HTLV-IIA, with which Si-IIA was established, but not by HTLV-II or well known oncogenic viruses from monkeys, although we can not rule out the possibility that HTLV-II might have induced lymphoma together with a co-factor such as deterioration of the host immunity by cytopathic effects on T cells.[23] Lymphoma induction experiments with IL-2-dependent HTLV-IIA could not be performed because of failure to recover stocked HTLV-IIA.

Malignant lymphomas induced by Si-IIA were clinically acute, fulminant and distributed in many organs, most frequently in the spleen, liver, kidneys and lymph nodes. There seemed to be no correlation between the number of cells inoculated (1×10^6 to 1×10^8) and survival time (62 to more than 172 days) of tumor-bearing rabbits. The reason for such a variable survival period is not known, but rabbits suffering from bronchopneumonia associated with pulmonary infiltration of lymphoma cells showed shorter survival periods than those without pneumonia. The apparent susceptibility and morphology of the induced disease were not altered by injection of peptide and complete Freund's adjuvant. Histologically, most of them were classified as diffuse, large cell type or mixed type non-Hodgkin's lymphoma. Only two cases (R-D and R-b) showed Hodgkin-like features. Hodgkin-like features as well as diffuse large cell lymphoma were observed in one case (R-D), suggesting polyclonal development of malignant lymphoma. Lymphoma cell clusters were usually accompanied with non-neoplastic neutrophils, and abscess-like central necrosis was frequently observed in large tumor masses. The phenotype of lymphoma cells was usually that of T cells except for Hodgkin-like lymphoma cells, which expressed only CD45. Leukemic change of malignant lymphoma was suspected only in one case (R-26) with 10% atypical lymphocytes but hematogenous metastases were suggested by perivascular invasion of lymphoma cells, intravascular atypical lymphocytes observed histologically in the tissues and cell line establishment from peripheral leukocytes of the tumor-bearing rabbit (R-D). Induced lymphomas

were usually lethal to host rabbits but regression was suggested in one case (R-a) showing complete tumor necrosis with marked calcification. Long-term prognosis and serial observation of the lymphoma development in this model must be further studied. Rabbits without susceptibility or those overcoming the disease would afford an opportunity to study host resistance to this unknown oncogenic agent.

Of five irradiated cell lines which were inoculated in 13 rabbits, only Ra-SLN cells induced malignant lymphoma in 1 of 3 rabbits. These findings indicate that only a few of the cell lines established may produce infectious and oncogenic viruses, which were different from already known oncogenic viruses, although an unknown provirus genome might possibly be integrated.

We plan to establish rabbit cell lines producing only the causative agent and having a high tumorigenicity, and to isolate and characterize this unknown causative factor (probably a virus) unrelated to HTLV-II. The host range of this factor must also be determined. This new lymphoma model of Japanese white rabbits should be very useful for the study of the pathogenesis, development or therapy of malignant lymphoma, including Hodgkin's disease.

ACKNOWLEDGMENTS

We are grateful to Mr. T. Zohta, Mr. T. Suzuki, Ms. M. Shiotani and Ms. Y. Emi for their skillful technical assistance.

(Received February 24, 1994/Accepted May 11, 1994)

REFERENCES

1) 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).
2) Rosenblatt, J. D., Golde, D. W., Wachsman, W., Giorgi, J. V., 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).
3) Kalyanaraman, V. S., Narayanan, R., Feorino, P., Ramsey, R. B., Palmer, E. L., Chorda, T., McDougal, S., Getchell, J. P., Holloway, B., Harrison, A. K., Cabradilla, C. D., Telfer, M. and Evatt, B. Isolation and characterization of a human T cell leukemia virus type II from a hemophilia-A patient with pancytopenia. *EMBO J.,* **4,** 1455–1460 (1985).
4) 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).
5) Page, J. B., Lai, S., Chitwood, D. D., Klimas, N. G., Smith, P. C. and Fletcher, M. A. HTLV-I/II seropositivity and death from AIDS among HIV-I seropositive intravenous drug users. *Lancet,* **335,** 1439–1441 (1990).
6) Kira, J., Koyanagi, Y., Hamamado, T., Itoyama, Y., Yamamoto, N. and Goto, I. HTLV-II in patients with HTLV-I-associated myelopathy. *Lancet,* **338,** 64–65 (1991).
7) Ijichi, S., Zaninovic, V., Leon-S., F. E., Katahira, Y., Sonoda, S., Miura, T., Hayami, M. and Hall, W. W. Identification of human T cell leukemia virus type IIb infection in the Wayu, an aboriginal population of Colombia. *Jpn. J. Cancer Res.,* **84,** 1215–1218 (1993).
8) Loughran, T. P., Jr., Coyle, T., Sherman, M. P., Starkebaum, G., Ehrlich, G. D., Ruscetti, F. W. and Poiesz, B. J. Detection of human T-cell leukemia/lymphoma virus, type II, in a patient with large granular lymphocyte leukemia. *Blood,* **80,** 1116–1119 (1992).
9) Zucker-Franklin, D., Hooper, W. C. and Evatt, B. L. Human lymphotropic retroviruses associated with mycosis fungoides: evidence that human T-cell lymphotropic virus type II (HTLV-II) as well as HTLV-I may play a role in the disease. *Blood,* **80,** 1537–1545 (1992).
10) Miyamoto, K., Tomita, N., Ohtsuki, Y. and Kitajima, K. Detection of provirus in an HTLV-II producer CD8+ T cell line by polymerase chain reaction combined with digoxigenin-ELISA method. *Jpn. J. Cancer Res.,* **81,** 313–316 (1990).
11) Miyamoto, K., Tomita, N., Hayashi, K., Ohara, N., Akagi, T., Ohtsuki, Y., Goda, N., Shirai, K., Okazaki, Y. and Kitajima, K. Isolation of HTLV-II and establishment of HTLV-II transformed T-cell lines having subtype a. *Igakunoayumi,* **164,** 657–658 (1993) (in Japanese).
12) Miyamoto, K., Tomita, N., Hayashi, K. and Akagi, T. Transformation of animal cells with human T-cell leukemia virus type II. *Jpn. J. Cancer Res.,* **81,** 720–722 (1990).
13) Hayashi, K., Ohara, N., Fujiwara, K., Aoki, H., Jeon, H, J., Takahashi, K., Tomita, N., Miyamoto, K. and Akagi, T. Co-expression of CD4 and CD8 associated with elevated interleukin-4 in a cord T cell line derived by coculturing normal human cord leukocytes and an HTLV-II-producing simian leukocyte cell line (Si-IIA). *J. Cancer Res. Clin. Oncol.,* **119,** 137–141 (1993).
14) Ohara, N., Hayashi, K., Miyamoto, K., Tomita, N., Fujiwara, K., Kondo, E., Takahashi, K., Ohtsuki, Y. and Akagi, T. A human T cell line with an abnormal trisomy 2 karyotype established by coculture of peripheral lymphocytes with an HTLV-II-infected simian leukocyte cell line. *Acta Pathol. Jpn.,* **43,** 237–243 (1993).
15) Hayashi, K., Ohara, N., Fujiwara, K., Jeon, H. J., Koirala, T. R., Takahashi, K., Akagi, T., Tomita, N. and

Miyamoto, K. HTLV-II-specific antisera raised in rabbits immunized with a synthetic peptide of HTLV-II envelope protein. *Virchows Arch. B,* **63,** 37–41 (1992).
16) Ohara, N., Hayashi, K., Takahashi, K., Miyamoto, K. and Akagi, T. A novel monoclonal antibody specifically reactive with human T-lymphotropic virus type-II (HTLV-II) envelope protein. *Int. J. Cancer,* **54,** 858–861 (1993).
17) Miyamoto, K., Tomita, N., Ishii, A., Nonaka, H., Kondo, T., Tanaka, T. and Kitajima, K. Chromosome abnormalities of leukemic cells in adult patients with T-cell leukemia. *J. Natl. Cancer Inst.,* **73,** 353–362 (1984).
18) Shimotohno, K., Takahashi, Y., Shimizu, N., Gojobori, T., Golde, D. W. and Chen, I. S. Y. 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).
19) Cockerell, G. L., Weiser, M. G., Rovnak, J., Wicks-Beard, B., Roberts, B., Post, A., Chen, I. S. Y. and Lairmore, M. D. Infectious transmission of human T-cell lymphotropic virus type II in rabbits. *Blood,* **78,** 1532–1537 (1991).
20) Fleckenstein, B. and Desrosiers, R. C. *Herpesvirus saimiri* and *Herpesvirus ateles. In* "Herpesviruses," Vol. 1, ed. B. Roizman, pp. 253–331 (1982). Plenum Press, New York and London.
21) Fujimoto, K., Terato, K., Miyamoto, J., Ishiko, H., Fujisaki, M., Cho, F. and Honjo, S. Establishment of a B-lymphoblastoid cell line infected with Epstein-Barr-related virus from a cynomolgus monkey *(Macaca fascicularis). J. Med. Primatol.,* **19,** 21–30 (1990).
22) Hayami, M. Simian T-cell leukemia virus (STLV-I) — HTLV-I-related virus in non-human primates. *Gann Monogr. Cancer Res.,* **39,** 247–256 (1992).
23) Miyamoto, K., Kamiya, T., Minowada, J., Tomita, N. and Kitajima, K. Transformation of CD8+ T-cells producing a strong cytopathic effect on CD4+ T-cells through syncytium formation by HTLV-II. *Jpn. J. Cancer Res.,* **82,** 1178–1183 (1991).