Jpn. J. Cancer Res. (Gann), 79, 335–341 ; March, 1988

# Adult T Cell Leukemia-like Disease Experimentally Induced in Rabbits

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**An HTLV-I-transformed T cell line, obtained from the peripheral blood of a virus-infected (B/J × Chbb:HM) F1 rabbit, was able to kill syngeneic newborn rabbits within 7 days, when inoculated intraperitoneally at a dose of $1 \times 10^8$ cells. Inoculation of $1 \times 10^7$ cells killed or rendered moribund 50% of inoculated animals, while surviving animals exhibited cell-mediated cytotoxic activities against the transformed cells. The peripheral blood leukocyte counts increased in all surviving animals, in association with appearance of abnormal lymphocytes with convoluted or lobulated nuclei. Pathological examination of animals that died one week post-inoculation revealed no tumors in the abdominal cavity, but accumulation of ascites containing abnormal lymphocytes. Histological examination showed leukemic infiltration in the liver, lungs, spleen and mesenteric lymph nodes. The same cell line was also able to kill syngeneic adult rabbits in 8–10 days when inoculated intravenously, but not intraperitoneally, at a dose of $1 \times 10^8$ cells. Leukemic infiltration was observed in the major organs of these animals. Adult animals which were already virus carriers were resistant to this lethal inoculation. This rabbit ATL-like disease may prove to be useful as an experimental model for acute adult T cell leukemia.**

Key words: Adult T cell leukemia — Animal model — Rabbit — HTLV-I

Adult T cell leukemia (ATL)[1] is a malignant hematological disorder, which has been etiologically linked to infection with human T lymphotropic virus type I (HTLV-I).[2] This disease can be classified into four types; smoldering, chronic, crisis and acute, according to the clinical symptoms and disease course, the prototype being the acute type.[3] Patients with acute ATL hardly respond to therapy, and have an extremely poor prognosis,[4] most dying shortly after diagnosis, with a median survival time of 4.2 months. Establishment of an animal model is an urgent requirement to enable studies into the prevention and treatment of this disease.

We recently succeeded in the induction in rabbits of a preleukemic stage of an ATL-like disease, by neonatal inoculation of syngeneic HTLV-I-transformed cells.[5] These cells did not kill the inoculated animals, but induced leukocytosis associated with the appearance of abnormal lymphocytes in the peripheral blood, and with leukemic infiltration into the major organs. We now report the induction of a fulminant ATL-like disease in adults as well as in newborn rabbits, by inoculation of a newly established HTLV-I-transformed T cell line.

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## MATERIALS AND METHODS

**Rabbits** Rabbits of two inbred strains, B/J and Chbb:HM, were originally derived from the Jackson Laboratory (Bar Harbor, Maine) and Dr. Karl Thomae GmbH (Biberach, West Germany), respectively.[6] Their F1 hybrids were bred in our laboratory and used for the present experiments.

**Cell Line** The HTLV-I-transformed T cell line, F647a, was established from a two-month-old female F1 rabbit, which had been neonatally infected with the virus, as described previously.[5] In brief, peripheral blood lymphocytes (PBL) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, and in the absence of T cell growth factor (TCGF). The established cell line had the karyotype of a normal female rabbit, and was positive for the HTLV-I p19 antigen, the pan T cell marker antigen and the Ia antigen, all three being detectable by indirect immunofluorescence using their respective monoclonal antibodies.

***In vivo* Inoculation of Transformed Cells and the Establishment of Cell Lines from Cell-inoculated Animals** HTLV-I-transformed cells were inoculated intraperitoneally into 3-day-old animals, and intravenously or intraperitoneally into adult animals. Some of these animals were killed 1–4 weeks post-inoculation, and their whole blood was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and 10% TCGF (JIMRO, Takasaki). Cell lines thus established were examined for the viral p19 antigen and the pan T cell marker by indirect immunofluorescence, and for integrated provirus genomes by Southern hybridization using the pMT2/65 probe (kindly provided by Dr. Robert C. Gallo).

**Virus-carrier Rabbits** Newborn F1 hybrids were inoculated subcutaneously with the HTLV-I-transformed T cel line derived from the Chbb:HM rabbit described previously,[5] and were used for experiments 8–15 months later. These animals were seropositive for anti-HTLV-I antibody, and possessed HTLV-I-infected cells in their peripheral blood, as shown by establishment of HTLV-I positive T cell lines.

**Cytotoxicity Tests** Spleen cell cytotoxic activity against $^{51}$Cr-labeled F648b cells[5] was assayed in plastic tubes according to the method described.[7] In brief, $5\times10^5$ labeled cells were mixed in 0.55 ml aliquots with spleen cells at 3 different ratios, and were allowed to react at 37° for 4 hr. The radioactivity in the supernatant was then counted and the percent specific lysis was calculated using the formula:

$$\frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100$$

where spontaneous release was determined with or without normal spleen cells present, and experimental release was that obtained in the presence of the effector cells being tested. Maximal release is the value obtained after freeze-thawing of target cells. Spontaneous release obtained without normal spleen cells was similar to that obtained in the presence of these cells and was less than 20% of maximal release. The cytotoxic activity of the serum antibody was similarly assayed in tubes, by adding $5\times10^5$ labeled cells in 50 $\mu$l aliquots to 0.5 ml of serum which had been diluted 1:10 in RPMI 1640 medium supplemented with 10% fresh normal rabbit serum. The mixture was allowed to react at 37° for 1 hr, the radioactivity in the supernatant was counted, and the percent specific lysis was calculated in the same way as for the cell-mediated cytolysis. Serum anti-HTLV-I antibody titers were determined by indirect immunofluorescence[8]; HUT 102 and HPB-ALL cells were used as virus-positive and virus-negative cells, respectively.

## Results and Discussion

**ATL-like Disease in Newborn Animals** Thirteen newborn F1 hybrids were inoculated intraperitoneally with transformed cells: 3 (rabbits Nos. 740, 745 and 746) received 5–10 $\times10^7$ cells, 8 (rabbits Nos. 741, 742, 747, 748, 756, 763, 766 and 767) received $1\times10^7$ cells and 2 (rabbit Nos. 743 and 744) received $2\times10^6$ cells. All 3 animals of the first group and 4 out of the 8 of the second group died or became moribund on days 5–10; the rest of the animals survived. Autopsy of the animals which were killed by the inoculation revealed no tumors in the abdominal cavity, but accumulation of 5–10 ml of ascites, containing abnormal lymphocytes with convoluted or lobulated nuclei. In immunofluorescence studies, these cells stained positively with anti-p19 monoclonal antibody. Histological examination showed typical leukemic infiltration in the lungs, liver, spleen and mesenteric lymph nodes (Fig. 1). *In vitro* cultivation of the ascites, as well as of peripheral blood, led to the establishment of cell lines within 2 weeks.

The 4 surviving animals in the second group were killed 2–4 weeks post-inoculation, and were examined histologically and immunologically. Establishment of cell lines from the peripheral blood required more than 2 weeks *in vitro* cultivation. White blood cell counts in these animals were higher than those in normal control animals, ranging from 12 to $18\times10^3$ cells/mm$^3$, as against normal counts of 4–$6\times10^3$/mm$^3$. Abnormal lymphocytes were found to account for 3–5% of the total, and were negative for HTLV-I p19 antigen at first, but became positive during *in vitro* cultivation. Upon autopsy, neither tumors nor ascites could be observed in the abdominal cavity, but histological examination showed infiltration of medium-sized leukemic cells in the lungs, liver, spleen and kidneys, similar to observations made previously in 3- to 5-week-old animals neonatally inoculated with other HTLV-I-transformed cell lines.[5]

Southern blot hybridization of *Eco*RI digests of DNA from ascites cells and the established cell lines was carried out using an HTLV-I probe (Fig. 2). Following inoculation, the F647a cell's monoclonal integration pattern of HTLV-I provirus was conserved in

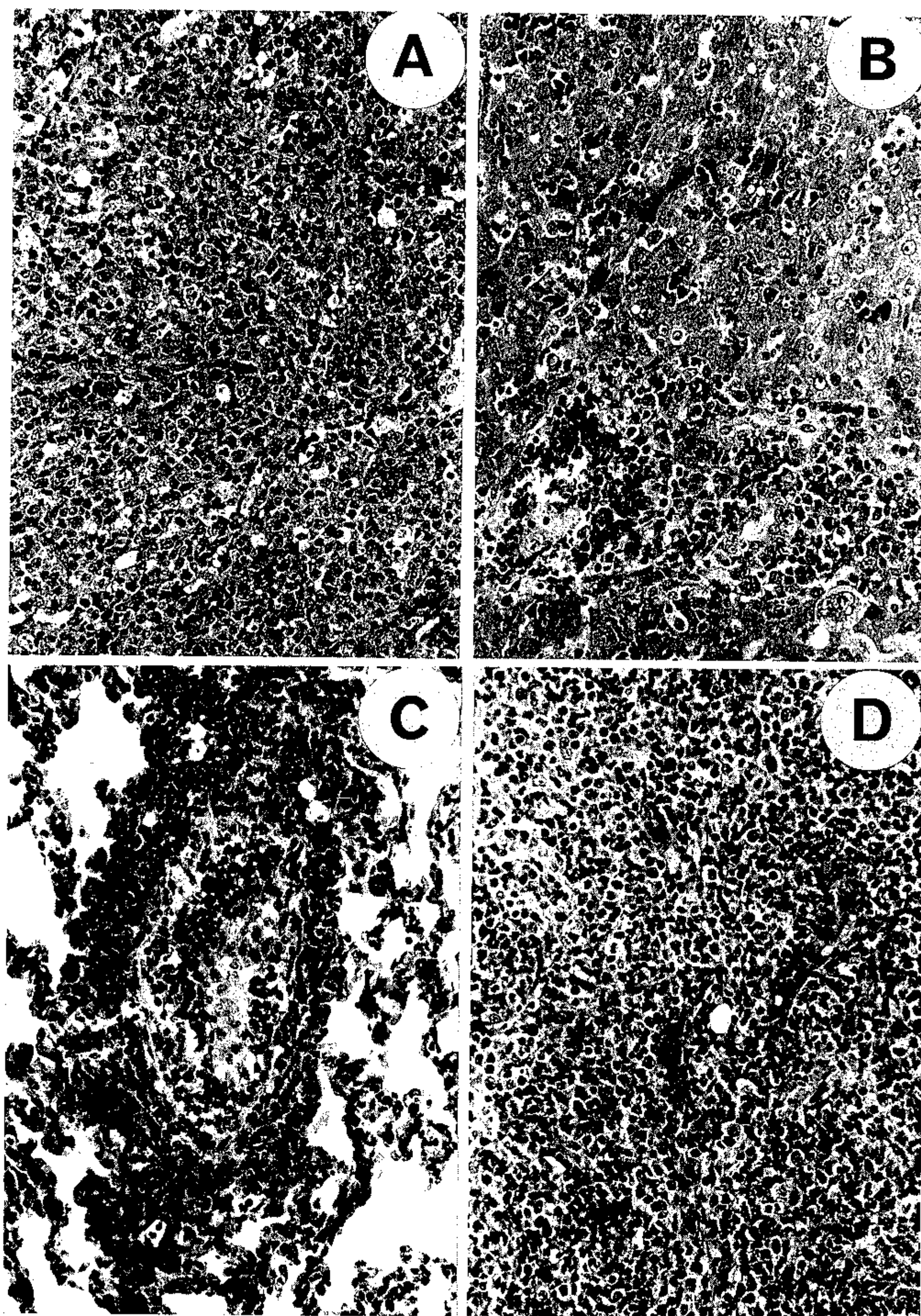


Fig. 1. Leukemic infiltration in major organs of newborn rabbit No. 741, which died 7 days post-inoculation. (A), Extensive proliferation of abnormal lymphoid cells in the white pulp of the spleen. Germinal centers are absent. (B), Portal triads of the liver are occupied by pleomorphic lymphoid cells with mitotic activity. Extramedullary hematopoiesis is also observed. (C), Lymphoid cell infiltration in perivascular lymph channels and in the interstitium of the lung. (D), The architecture of the lymph node is obliterated by diffuse infiltration of lymphoid cells. Stain, hematoxylin and eosin; magnification, ×200.

both the ascites cells and the cell lines established from blood one week post-inoculation, suggesting that the major leukemic cells in animals at this age were direct progeny of the inoculated cells. Cell lines established from surviving animals 2–4 weeks post-inoculation showed different integration patterns, suggesting that most of these were not progeny of the inoculated cells, but were probably of recipient origin. These results are consistent with previous data obtained with other cell lines.[5]

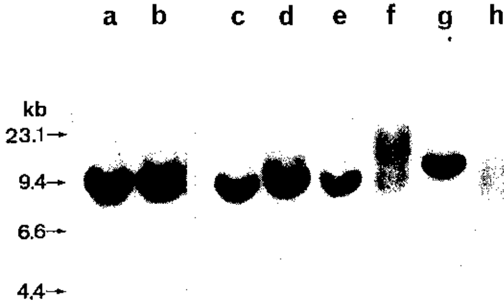


Fig. 2. Southern hybridization of DNA from inoculum cells, ascites cells and cell lines established from inoculated animals. Inoculum cell (lanes a and c), ascites cell from rabbit No. 741 (lane b), cell lines from rabbits No. 741 (lane d) and No. 746 (lane e) at 1 week post-inoculation, from rabbit No. 745 (lane f) at 2 weeks, and from rabbits No. 742 (lane g) and No. 744 (lane h) at 4 weeks post-inoculation.

Cytotoxicity tests were carried out for the serum and spleen cells of surviving animals, and the results are shown in Table I. Whereas the sera were not cytotoxic toward HTLV-I-transformed cells, spleen cells were cytotoxic against these cells: this activity was detected at 3 weeks of age, when no anti-virus antibody could be found in the serum by indirect immunofluorescence. This cell-mediated cytotoxic activity may have played a role in preventing the development of the ATL-like disease in these animals. Newborn rabbits have immature immune functions, and the above data suggest that leukemic death of inoculated neonates could occur only if the inoculated cells were able to proliferate sufficiently before specific killer cells had been generated.

**ATL-like Disease in Adult Animals** The leukemia-inducing activity of the transformed cell line in adult animals was examined by inoculating $1\times10^8$ cells into two animals (rabbits Nos. 726 and 727) intraperitoneally, and into two others (rabbits Nos. 610 and 611) intravenously. Three HTLV-I carrier animals (rabbits Nos. 601, 639 and 648) were also inoculated intravenously with the same number of cells. The results showed that this cell line was able to kill adult syngeneic

Table I. Anti-HTLV-I Immune Responses in Surviving Rabbits Neonatally Inoculated with Transformed Cells

| Rabbit No. | Number of inoculum cells | Age at sacrifice (wk) | Cell-mediated cytolysis[a] E/T ratio[b] | (%) | Antibody-mediated cytolysis[a] (%) | Anti-virus antibody titer[c] |
|---|---|---|---|---|---|---|
| 756 | $1\times10^7$ | 3 | 10:1 | 16.7 | 0 | <1:10 |
| | | | 5:1 | 14.0 | | |
| | | | 2.5:1 | 8.4 | | |
| 766 | $1\times10^7$ | 5 | 10:1 | 24.6 | 0 | 1:160 |
| | | | 5:1 | 20.9 | | |
| | | | 2.5:1 | 14.1 | | |
| 743 | $2\times10^6$ | 12 | 10:1 | 0 | 0 | 1:10 |
| | | | 5:1 | 0 | | |
| | | | 2.5:1 | 0.6 | | |

*a*) $^{51}Cr$ release assay.
*b*) Effector (spleen cells)/target (F648b cell line) ratio.
*c*) Indirect immunofluorescence.

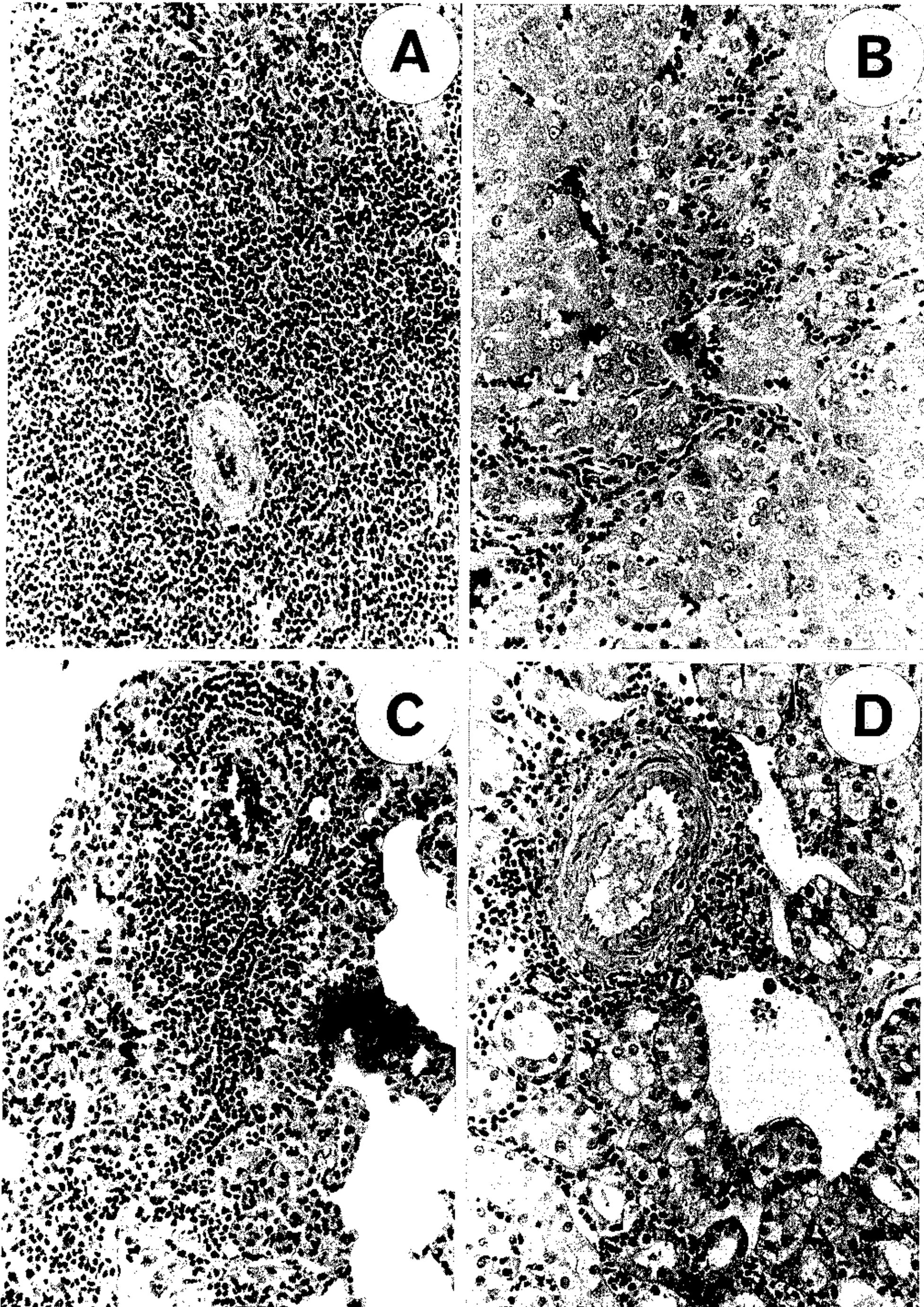


Fig. 3. Leukemic infiltration in the major organs of adult rabbit No. 611. (A), In the white pulp of the spleen, monomorphic lymphoid cell infiltration surrounds the central artery. (B), Infiltration into the portal spaces of the liver by small abnormal lymphocytes with scant cytoplasm and irregular nuclei. Mitotic figures can be observed. (C), Interstitial infiltration in the lung. (D), Interstitial infiltration in the kidney. Stain, hematoxylin and eosin; magnification, ×200.

Table II. Anti-HTLV-I Immune Responses in Virus-carrier Rabbits Inoculated with Transformed Cells

| Rabbit No.[a] | Cell-mediated cytolysis[b] E/T ratio[c] (%) | | Antibody-mediated cytolysis[b] (%) | Anti-virus antibody titer[d] |
|---|---|---|---|---|
| 601 | 10:1 | 13.2 | 5.8 | 1:160 |
| | 5:1 | 8.5 | | |
| | 2.5:1 | 6.0 | | |
| 639 | 10:1 | 3.3 | 18.8 | 1:160 |
| | 5:1 | 1.6 | | |
| | 2.5:1 | 0.0 | | |
| 648 | 10:1 | 10.6 | 7.6 | 1:160 |
| | 5:1 | 7.8 | | |
| | 2.5:1 | 4.5 | | |

*a*) Examined 3 months post-inoculation.
*b*) $^{51}$Cr release assay.
*c*) Effector (peripheral blood leukocytes)/target (F648b cell line) ratio.
*d*) Indirect immunofluorescence.

animals 8–10 days following intravenous, but not intraperitoneal inoculation. Microscopic examination revealed leukemic infiltration in the major organs of the rabbits inoculated intravenously, but not of those inoculated intraperitoneally (Fig. 3). These infiltrating cells were smaller than those observed in newborn animals. It was not clarified whether these cells were the progeny of the inoculum or were of recipient origin. Cell lines were not established from the PBL of these animals. These results indicate that even the mature immune functions of the adult animals were not sufficiently effective in resisting massive inoculations of leukemic cells directly into the blood stream. However, all three virus-carrier animals were resistant to this cell line's lethal challenge, and mounted immune responses against it (Table II). This resistance may be due to the same specific immunity as was observed in surviving neonatally inoculated animals. One of the carriers was the animal from which the F648a and b cell lines, used in the previous study,[5] had been established. In that study, these two cell lines induced a preleukemic state following neonatal inoculation. Nevertheless, this animal has not developed any overt disease after more than eight months, suggesting that the development of the disease was suppressed in the carrier state and following lethal challenge.

HTLV-I can infect and transform T cells not only from humans but also from animals such as monkeys,[9] rabbits[10] and rats,[11] making it possible to establish experimental models of ATL in these animals. The models thus far reported, however, have differed from the human disease. On the other hand, the present model in rabbits closely resembles human acute type of ATL in that typical leukemic infiltration is observed in major organs, leading to a fulminant disease. Although the mechanism of the disease onset seems to be different between human ATL and the ATL-like disease in rabbits, this animal model may be useful for studies on the prevention and treatment of leukemic infiltration in the human disease.

## Acknowledgments

The authors are grateful to Dr. Robert C. Gallo for providing monoclonal anti-p19 antibody and pMT2/65 probe, and to Dr. Thomas J. Kindt for providing monoclonal antibodies against rabbit Ia and pan T cell marker. The authors wish to thank Ms. K. Kumagai for her technical assistance. This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture, Japan.

*(Received Oct. 27, 1987/Accepted Jan. 13, 1988)*

## References

1) Uchiyama, T., Yodoi, J., Sagawa, K., Takatsuki, K. and Uchino, H. Adult T-cell leukemia: clinical and hematologic features of 16 cases. *Blood*, **50**, 481–492 (1977).
2) Yoshida, M., Miyoshi, I. and Hinuma, Y. Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease. *Proc. Natl. Acad. Sci. USA*, **79**, 2031–2035 (1982).
3) Kawano, F., Yamaguchi, K., Nishimura, H., Tsuda, H. and Takatsuki, K. Variation in the clinical courses of adult T-cell leukemia. *Cancer*, **55**, 851–865 (1985).
4) Shimoyama, M., Yunoki, K., Ichimaru, M., Ohta, K. and Ogawa, M. Combination chemotherapy with vincristine, cyclophosphamide (Endoxan), prednisolone and adriamycin (VEPA) in advanced adult non-Hodgkin's lymphoid malignancies: relation

between T-cell or non T-cell phenotype and response. *Jpn. J. Clin. Oncol.*, **9** (Suppl), 397–406 (1979).

5) Seto, A., Kawanishi, M., Matsuda, S., Ogawa, K., Eguchi, T. and Miyoshi, I. Induction of preleukemic stage of adult T cell leukemia-like disease in rabbits. *Jpn. J. Cancer Res. (Gann)*, **78**, 1150–1155 (1987).
6) Seto, A., Fu, N., Matsuda, S., Eguchi, T., Miyoshi, I. and Ito, Y. Introduction of inbred rabbits into research on viral tumors. *In* "Cancer Cells 5," ed. B. Steinberg, J. Brandsma and L. Taichman, pp. 367–371 (1987). Cold Spring Harbor Laboratory, New York.
7) Brunner, K. T., Engers, H. D. and Cerottini, J.-C. The $^{51}$Cr release assay as used for the quantitative measurement of cell-mediated cytolysis *in vitro*. *In "In vitro* Methods in Cell-mediated and Tumor Immunity," ed. B. R. Bloom and J. R. David, pp. 423–428 (1976). Academic Press, New York.
8) 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).
9) Miyoshi, I., Taguchi, H., Fujishita, M., Yoshimoto, S., Kubonishi, I., Ohtsuki, Y., Shiraishi, Y. and Akagi, T. Transformation of monkey lymphocytes with adult T-cell leukemia virus. *Lancet*, **1**, 1016 (1982).
10) Miyoshi, I., Yoshimoto, S., Taguchi, H., Kubonishi, I., Fujishita, M., Ohtsuki, Y., Shiraishi, Y. and Akagi, T. Transformation of rabbit lymphocytes with T-cell leukemia virus. *Gann*, **74**, 1–4(1983).
11) Tateno, M., Kondo, N., Itoh, T., Chubachi, T., Togashi, T. and Yoshiki, T. Rat lymphoid cell lines with human T cell leukemia virus production. *J. Expl. Med.*, **159**, 1105–1116 (1984).