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Frequent Development of Murine T-Cell Lymphomas with TcRα/β^+, CD4$^-$/8$^-$ Phenotype after Implantation of Human Inflammatory Breast Cancer Cells in BALB/c Nude Mice

Hiro Wakasugi,[1,6] Kotaro Koyama,[2] Motomichi Gyotoku,[3] Masataka Yoshimoto,[4] Setsuo Hirohashi,[5] Takashi Sugimura[2] and Masaaki Terada[2]

[1]*Section of Studies of Host-Immune Response,* [2]*Genetics Division,* [3]*Growth Factor Division and* [5]*Pathology Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104 and* [4]*Division of Surgery, Cancer Institute Hospital, 1-37-1 Kami-Ikebukuro, Toshima-ku, Tokyo 170*

Tumors developed quite frequently in some of the visceral organs, including spleen and liver, in BALB/c nude mice upon subcutaneously xenografting surgical specimens from five different inflammatory breast cancer patients. All of these tumors developed within two and a half months to one year after the subcutaneous inoculation of surgical specimens. From these tumors, five independent transplantable tumors, including tMK-2, tHK-1, tYK-1, tYK-2 and tTY-1 have been established. Chromosome analysis, morphologic studies by light and electron microscopy and phenotype analysis indicated that these tumors are of mouse origin. The tMK-2 tumor was highly metastatic to the spleen and liver when it was subcutaneously transplanted into the right scapular region. In addition, the region where the tMK-2 tumor cells were subcutaneously inoculated showed an apparently inflammatory process represented by erythema. After subcutaneous inoculation into the right scapular region, tHK-1, tYK-1, 2, and tTY-1 tumors also metastasized to some of the visceral organs, including spleen and liver. From these tumors, *in vitro* cell lines were established. The cells grew in a stromal-cell dependent manner under *in vitro* culture conditions. The cells were again tumorigenic at the inoculated region and metastasized to various organs, including liver and spleen, of BALB/c nude mice. Histological examination revealed that the tumors showed features of malignant lymphoma. Phenotypically, these five tumors expressed early T lymphocyte markers as revealed by anti-mouse anti-TcRα/β, anti-CD3, CD4 and CD8 monoclonal antibodies. To our knowledge, these cell lines are the first T-cell lines showing the phenotype of extrathymically differentiated T-cells in the liver.

Key words: Inflammatory breast cancer — *In vivo* transformation — T-Cell lymphoma — Extrathymic pathway

The incidence of cancer of the breast has recently been increasing throughout the world. The progress of breast cancer treatment, including surgical, hormonal and chemo-therapies, has resulted in an excellent 10-year survival rate.[1] Inflammatory breast cancer, however, is extremely malignant and remains resistant to therapeutic treatment, although its incidence is less than 5% of that of breast cancer in general. This cancer is striking for the following reasons. Firstly, it is associated with inflammatory features, including erythema and pain, but there is no bacterial infection. Secondly, the patient has an extremely poor prognosis and dies within several to ten months with systemic metastasis.[2] To elucidate the biological properties of this type of malignant breast cancer, we endeavored to establish inflammatory breast cancer cell lines by inoculating surgical specimens into nude mice.

We here report the development of 5 independent malignant T-cell lymphomas in nude mice upon xenografting of 5 different surgical specimens of inflammatory breast cancer patients. It is well known that human tumors rarely induce the transformation of mouse stromal cells into fibrosarcomas when they are xenografted in nude mice.[3–8] The incidence of lymphoma induction and the characteristics of the induced lymphomas still remain unclear. For instance, frequent induction of lymphomas in antigenically stimulated athymic mice has been reported.[9] There are also some examples of the transformation of the host effector cells, lymphocytes.[10,11] This is the first report showing that lymphomas of T-cell origin with TcRα/β^+, CD4$^-$/8$^-$ phenotype are induced by inoculation of human tumors into nude mice with quite high incidence.

MATERIALS AND METHODS

Tumor specimens Tumor specimens from patients with inflammatory breast cancers were obtained immediately after surgery and processed as follows. Tumor tissue was dissected free of necrotic areas, connective tissue and blood and rinsed several times in a cell culture medium composed of RPMI-1640 medium supplemented with

[6] To whom all correspondence should be addressed.

10% fetal calf serum (FCS) and antibiotics (penicillin 100 units/ml; streptomycin 100 μg/ml). The tissue was then cut into fragments of about 3 mm^3 using a sterile scalpel blade and inoculated subcutaneously into the abdominal wall of two to three BALB/c female nude mice.

Mice Five-week-old female BALB/c nude mice were obtained from Charles River Japan, Inc., Tokyo, and used at six weeks of age. For the tumorigenicity test, female BALB/c mice were also purchased from the same company.

Development and maintenance of *in vivo* tumor lines The 'discovery of tumor development' means an apparent enlargement of the abdomen accompanied with an enlargement of the spleen and liver or other visceral organs which can be easily seen as a mass through the abdominal wall or an apparent enlargement of lymph nodes in the submandibular, axillar and/or inguinal regions.

The tumor specimens from donor patient MK were subcutaneously inoculated into the abdominal wall in two mice. Two months after inoculation of the surgical specimens, the primary inoculated tumors in these two mice disappeared, and abdominal enlargement was seen. The first mouse died one month after the discovery of abdominal tumor development. We performed a necropsy and severe hepato-splenomegaly occupied almost all of the abdominal cavity. We could not perform a histological study because the visceral organs had not been stored under appropriate conditions. In the case of the second mouse, a tumor also developed and the mouse was killed and autopsied. Upon autopsy, diffuse hepatomegaly and splenomegaly with white disseminated foci were observed. Tissue fragments (3–5 mm^3) from the liver or spleen were independently inoculated subcutaneously into the right scapular region of BALB/c female nude mice. Tumors developed with abdominal enlargement in all of four mice two months after the inoculation, but not in the inoculated site, and again tumors in the liver or spleen were inoculated into nude nice. From the 3rd generation of the tumor-recipient mice, however, tumors developed in the inoculated site as well as in the liver and spleen in all mice, and these mice died within three weeks after inoculation. Hepato-splenomegaly was always observed after the subcutaneous inoculation of either liver or splenic tumors. The tumors in the liver or spleen developed sometimes diffusely and sometimes with white foci. A tumor derived from the liver was maintained *in vivo* for more than 10 passages (tumors in the liver were xenografted subcutaneously every three weeks in the right scapular region in female BALB/c nude mice) and designated as tMK-2.

One of two mice inoculated with surgical specimens from donor patient HK into the abdominal wall developed axillary and inguinal lymph node swellings as well as enlargement of the abdomen 8.5 months after the inoculation. The mouse was killed 12 months after xenografting. Pieces of liver were subcutaneously inoculated into the right scapular region of nude mice. The tumor from the liver was maintained for more than 10 passages as described for tMK-2, and was designated as tHK-1.

Two out of three mice inoculated subcutaneously with surgical specimens from donor patient YK into the abdominal wall developed tumors six and seven months after xenografting. One of these two mice developed a tumor, showing enlargement of the liver and spleen, accompanied with swelling of lymph nodes of the axillar and inguinal regions. This mouse was killed nine months after xenografting. The tumor in the liver was maintained for more than 10 passages as described for tMK-2, and was designated as tYK-1. The second mouse was killed 11 months after xenografting and a tumor developed in the liver and spleen, with swelling of the uterus. The tumor that developed in the uterus was further inoculated into nude mice and the tumor that developed in the liver was maintained by serial passage as described for tMK-2, and was designated as tYK-2.

One out of three mice inoculated with a surgical specimen from donor patient KS in the abdominal wall developed tumors with enlargement of the abdomen and lymph nodes of the axillar and inguinal regions six months after inoculation. tKS-1 tumor, however, could not be established due to the sudden death of these mice for unknown reasons.

Tumor development was observed in one out of two mice 9 months after the subcutaneous inoculation of surgical specimens from donor patient TY into the abdominal wall, and this mouse was killed ten months after xenografting. Tumors developed in the liver and spleen accompanied with swelling of the mesenteric lymph nodes and the uterus. The tumors from the liver were further inoculated subcutaneously into nude mice and a tumor that developed in the liver after serial passages, as described for tMK-2, was designated as tTY-1.

Establishment of *in vitro* tumor cell line A mixed suspension of tumor cells and stromal cells of the liver or spleen was prepared from liver or spleen tumor which developed from subcutaneously transplanted tMK-2 tumor. After disaggregation with scissors and forceps, and pipetting of the tumor tissue, the cells were suspended in a cell culture medium and the suspensions were washed three times by gentle centrifugation at 800 rpm for five minutes. The cell pellet was resuspended in a cell culture medium. Cells were grown at 37°C in a humidified atmosphere of 5% CO_2. One week after the start of *in vitro* cell culture, the tumor cells began to grow only in the presence of stromal cells.

Chromosome analysis For karyotype studies of the *in vivo* and *in vitro* cell lines, the Giemsa G banding method was employed.[12]

Histological and electron microscopic examinations Tissue samples were fixed in 10% neutral buffered formalin, processed routinely, and embedded in paraffin. Sections (4 μM) stained with hematoxylin and eosin were examined by microscopy. For electron microscopy, tMK-2 tumor cells in suspension culture were fixed in 2% glutaraldehyde, postfixed in osmium tetroxide, and embedded in epoxy resin. Ultrathin sections were examined with an electron microscope (Nihon Denshi, JEM-1200EX, Tokyo).

Flow cytometry To examine ploidy, the sample cells were centrifuged and the pellets were prefixed with 70% ethanol. The cells were then suspended in propidium iodide hypotonic citrate and analyzed on a flow cytometer (FACScan, Becton Dickinson, Tokyo). Data are expressed as the DNA index (ratio of modal G1 DNA content of tumor cells versus diploid reference standard), which is used as a measure of ploidy. Human lymphocytes and mouse bone marrow cells were used as diploid controls for human and mouse cells, respectively.

For phenotype analysis, freshly isolated tumor cells from the liver were prepared by disaggregation with scissors and forceps, and pipetting. The cells were then suspended in culture medium and the suspensions were washed three times by gentle centrifugation at 800 rpm for five minutes. The cell pellet was resuspended in a standard culture medium. Mononuclear cells were isolated from parenchymal hepatocytes and Kupffer cells by Ficoll-Isopaque density gradient centrifugation at 2500 rpm for 30 min.[13] After centrifugation, the cells were washed three times by centrifugation at 800 rpm for five minutes and resuspended in a standard culture medium. For the isolation of the *in vitro*-cultured lymphoma cells, cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 1 h. The non-adherent cells to the culture flask were then collected. The cells were stained with fluorescein isothiocyanate (FITC)-labeled anti-mouse CD2, CD3, CD4, CD8 or Mac 1 monoclonal antibodies (MoAbs), phycoerythrin (PE)-labeled anti-mouse T-cell receptor (TcR)α/β, H-2K^d or Ig (κ) MoAb at 0°C for 30 min. The cells were washed twice with PBS and examined with a flow cytometer (Ortho Diagnostics Systems Co., FACScan, Tokyo). FITC- or PE-labeled irrelevant Ig MoAbs with the same isotype were used as a control. All MoAbs were purchased from Pharmingen, San Diego, CA.

DNA extraction and gel electrophoresis Preparation of mouse DNA and agarose gel electrophoresis were performed according to the established methods.[14]

RESULTS

Development of tumors in BALB/c nude mice and maintenance of tumor *in vivo* The time course of tumor development in nude mice is shown in Fig. 1. All surgical specimens from five (MK, HK, YK, KS and TY) donor patients induced tumors in nude mice. Altogether, tumor development was observed in seven out of twelve nude mice (overall incidence was 58%). As shown in Fig. 1, all tumors developed less than one year after inoculation. The earliest case was the tumor from donor patient MK, 2.5 months after xenografting. The latest case was the tumor development from donor patient TY, nine months after tumor inoculation. In the case of donor patient YK, tumors developed six and seven months after inoculation. In one out of three mice of donor patient KS, one mouse developed tumors six months after the inoculation. No

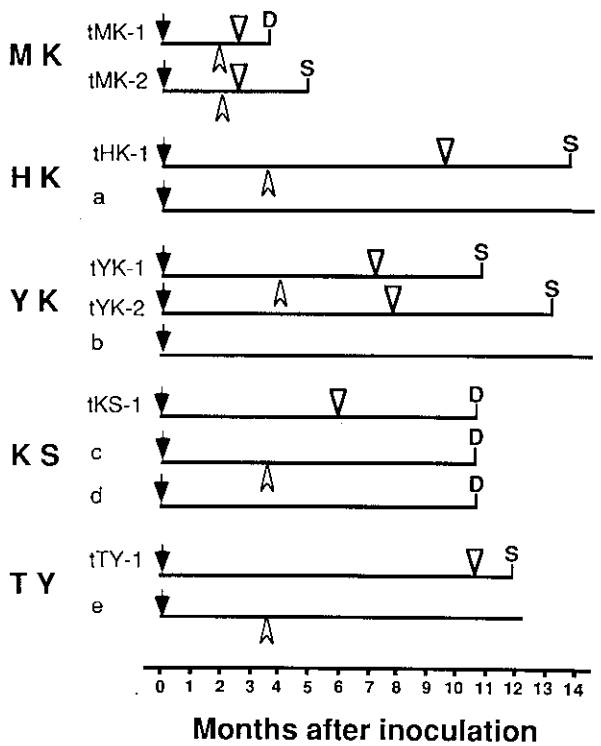

Fig. 1. Time course of tumor development after inoculation of inflammatory breast cancer. MK, HK, YK, KS and TY refer to the initials of donor patients with inflammatory breast cancer. The murine tumor cells that had been maintained *in vivo* and metastasized to the liver for more than ten passages were designated as tMK-1, tMK-2, tHK-1, tYK-1, tYK-2, tKS-1 and tTY-1, based on their origin. In a–e, the mice inoculated with the surgical specimens from the donor patients did not develop any visible tumors. Closed arrows (▼) show the time of inoculation of the surgical specimens. Open arrows (▽) indicate the discovery of tumor development. Upward arrows (△) show the time of disappearance of the subcutaneously inoculated surgical specimens. 'S' means the time when the nude mouse was killed for the next tumor passage as described in the text. 'D' means the death of the nude mouse.

Table I. Weights of Organs in Tumor-bearing and Control Mouse[a)]

Name of organ total	Tumor-bearing mouse[b)]	% of total weight	Control[c)]	% of weight
Total body weight (g)	29.38	—	28.6	—
Lung	0.27	0.92	0.22	0.77
Liver	3.40	11.6	1.95	6.8
Spleen	1.98	6.73	0.15	0.52
Kidney	0.64	2.18	0.68	2.38
Lymph nodes[d)]	1.09	3.71	—	—

a) Representative data from one of three independent measurements.
b) The tumor-bearing mouse had been killed 12 months after xenografting the primary surgical specimen of donor patient HK.
c) Control female BALB/c nude mouse 12 months after birth.
d) Lymph nodes of submandibular, axillar and inguinal regions.

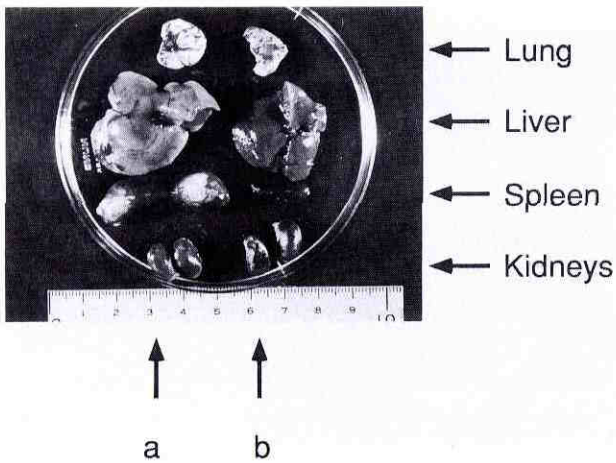

Fig. 2. Macroscopic findings of the organs of the nude mouse inoculated with inflammatory breast cancer. Photograph of each organ of a nude mouse implanted with inflammatory breast cancer of donor patient HK. Severe hepatomegaly with diffuse infiltration of the tumor cells and splenomegaly with white foci are seen. Each organ of the tumor-bearing mouse was compared to that of the control mouse. a, mouse implanted with the surgical specimen of donor patient HK; b, control mouse.

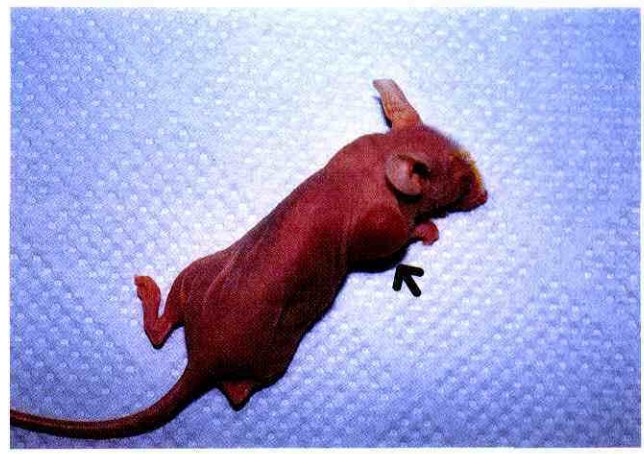

Fig. 3. Characteristic appearance of tumor at the site of subcutaneous inoculation of liver tumor induced by inflammatory breast cancer. The anesthetized nude mouse (eight-week old) was photographed two weeks after subcutaneous inoculation in the right scapular region of liver tumor induced by inflammatory breast cancer of donor patient MK, showing a tumor with erythema.

correlation between the time of disappearance of the inoculated tumor and that of development of the host tumors was apparent. The s.c.-inoculated surgical specimens did not show any growth after xenografting.

Fourteen nude mice were used as a control, including ten nude mice inoculated subcutaneously with 10^7 cells of established esophageal cancer cell lines per mouse (three mice with TE4 cells, one mouse with TE2 cells, one mouse with TE8 cells, two mice with TE10 cells and three mice with TE6 cells) and four nude mice with two surgical specimens from two different breast cancers of non-inflammatory type. They did not induce tumors of human or mouse origin during at least twelve months and ten months, respectively (H. Sakamoto, unpublished observations).

Macroscopic characteristics of the developed tumors The weight of each organ of a tumor-bearing nude mouse compared to a control nude mouse of the same age is shown in Table I (see also Fig. 2). The spleen was 13 times heavier than the control spleen, and the liver was 1.7 times heavier than the control liver. The white foci seen in the spleen in Fig. 2 were sometimes observed in the liver during serial passage of the tumors. The total weight of the submandibular, axillar and inguinal lymph

nodes was increased to 1.09 g, whereas that of the lymph nodes of the control mouse was less than 0.1 g.

As shown in Fig. 3, another feature of the tumor is an obvious inflammatory reaction around the subcutaneously inoculated tumor with erythema, suggesting that some factors involved in the inflammation were transferred from the surgical specimens of inflammatory breast cancer. Inflammatory reaction at the site of inoculation was also seen when the serially transplanted tMK-2 tumor cells were inoculated subcutaneously into nude mice.

Histological findings A photomicrograph of a section of the surgical specimen of donor patient MK is shown in Fig. 4-a. The primary tumor showed the histological features of infiltrating duct carcinoma of scirrhous type. Tumor cells were poorly differentiated, and were arranged in trabeculae, lacking glandular structures. Cancer stroma showed fibrous reaction with hyalinization and marked infiltration of inflammatory cells. Skin near the primary tumor (Fig. 4-b) showed infiltration of cancer cells to the epidermis and dilated dermal lymphatics, which are typically seen in "inflammatory carcinoma" of the breast. Intraductal spread and infiltration of cancer cells to adipose tissue were also observed.

Photomicrographs of sections of the tumors that developed in nude mice are shown in Fig. 4-c and 4-d. In the spleen, the tumor growth occupied the white pulp. Tumor cells resembled atypical lymphoid cells and were

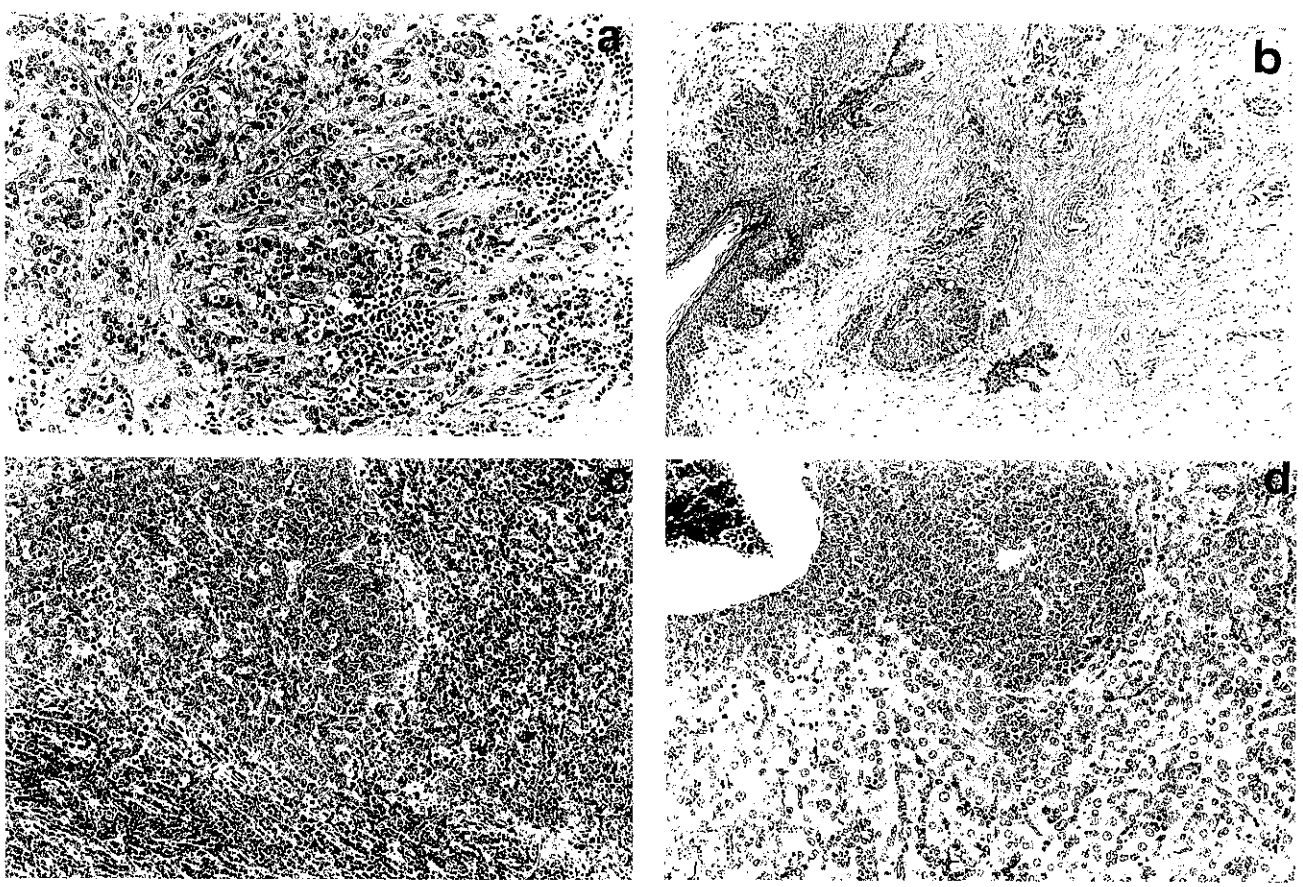

Fig. 4. Microscopic findings of the primary tumor of inflammatory breast cancer and the induced tumor in the nude mouse. a, ×250. Photomicrograph of a section of the surgical specimen of donor patient MK, showing typical breast cancer associated with apparent infiltrating ductal carcinoma of schirrous type, with infiltration of inflammatory exudative cells including multinuclear leukocytes, lymphocytes, enlargement of capillaries, development of collagen fibers and infiltration of the cancer cells to the lymphatic ducts, capillaries and skin. Cancer cells are of non-differentiated type with no adhesive capacity. b, ×125. Photomicrographs of a section of the subcutaneously developed tumor in a nude mouse. Small lymphoid tumor cells were infiltrating to the level of the epidermis and there is no isolation of tumor cells by a capsule. c, ×250. Section of enlarged spleen. There is mass infiltration of lymphoid tumor cells into white pulp. Stained with hematoxylin and eosin. d, ×250. Section of enlarged liver. Diffuse infiltration of lymphoid tumor cells in sinusoids and tumor formation surrounding central veins.

intermingled with macrophages (Fig. 4-c). In the liver, tumor cells infiltrated diffusely in sinusoids and formed tumors surrounding central veins and at portal spaces (Fig. 4-d). These histological features indicate that the tumor developed in mice is completely different from the primary tumor and can be diagnosed as a malignant lymphoma.

Electron microscopy Electron microscopic analysis revealed cells with very irregular nuclear appearance, such as lobulated, convoluted, mullberry and cerebriform (Fig. 5). The chromatin disappeared, with some condensation at the nuclear margin and scattered chromatin clumps and a prominent nucleolus. Cells with lobulated or cerebriform nucleus and prominent nucleolus were observed. The cytoplasm contained scanty organelles, including mitochondria, rough-surfaced endoplasmic reticulum, ribosomes, and myelin bodies. These findings are consistent with the morphological characteristics of T-cell lymphoma cells derived from an experimentally established cell line.

Karyotype analysis of developed tumor cells Chromosome analysis of the tumor cells was performed after 10 passages of tumor cells in nude mice. Examination for karyotype morphology of three hundred metaphases each derived from tMK-2 tumor in the liver, subcutaneously inoculated tMK-2 tumor and *in vitro*-cultured tMK-2 tumor cells showed that these tumor cells were exclusively of mouse origin and diploid. Representative Giemsa-banded karyotypes of the *in vitro*-cultured tMK-2 tumor cells are shown in Fig. 6. Chromosome analysis of tHK-1 and tYK-1 tumor cells also gave essentially the same results (data not shown). As shown in Table II, the values of the DNA index of tMK-2 and tHK-1 tumor cells were 1.16 and 1.05, respectively, again indicating that these tumor cells were diploid.

We also confirmed the origin of tumor cells by performing digestion of the high-molecular-weight DNA from the tMK-2 tumor cells. The electrophoretic pattern of the DNA from tumor cells corresponded predominantly to a mouse origin, and we could detect no human DNA pattern (data not shown).

Cultured tumor cell lines Attempts were made to transfer the tMK-2 tumor cells from nude mice to *in vitro* culture. From the liver or spleen tumor region of a

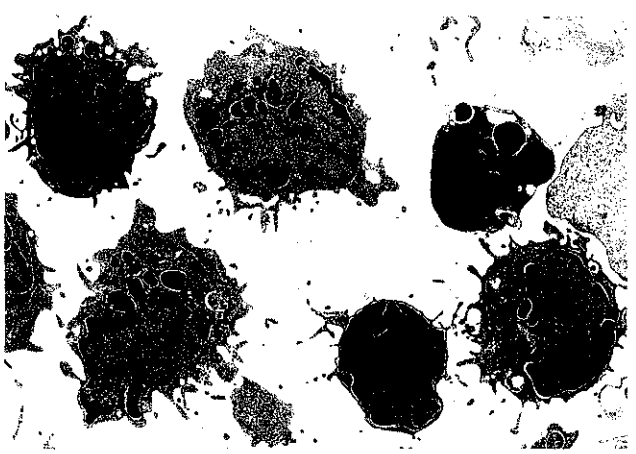

Fig. 5. Electron photomicrographs of *in vitro*-cultured tumor cells. ×7200. The cells show irregular nuclear appearance. The cytoplasm contains scanty organelles such as mitochondria, rough-surfaced endoplasmic reticulum, ribosomes, ceterioles and myelin bodies.

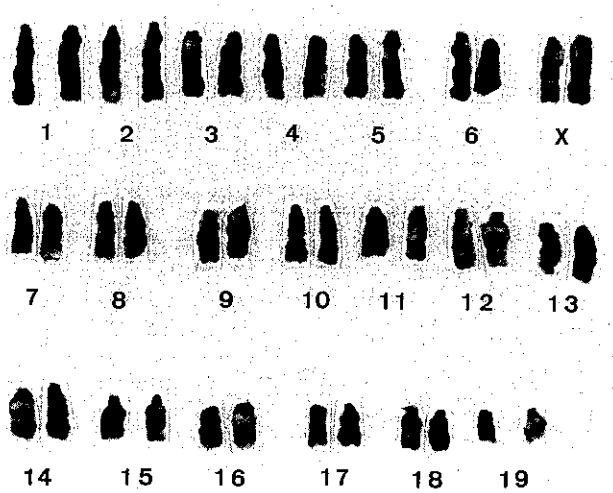

Fig. 6. Karyotype analysis of the induced tumor cells. Giemsa-banded karyotype analysis was performed on *in vitro*-cultured cells from the established tumor cells tMK-2 in a nude mouse. The number of chromosomes was 2N=40, indicating a normal karyotype of mouse origin. The karyotype analysis of the tMK-2 cells metastasized to the liver and inoculated subcutaneous tumor in the scapular region of nude mice showed essentially similar results.

Table II. Ploidy Analysis

	Peak channel	DNA index[a)]
Control[b)]	60	1.0
MK[c)]	70	1.16
HK[d)]	63	1.05

a) Refers to G1 peaks with 1 being diploid.
b) Cells from an adult BALB/c nude mouse.
c) Established tMK-2 tumor cells of the nude mouse xenografted with cancer cells from patient MK. Performed after a 1-month culture.
d) Established tHK-1 tumor cells of the nude mouse xenografted with cancer cells from patient HK. Performed after a 1-week culture.

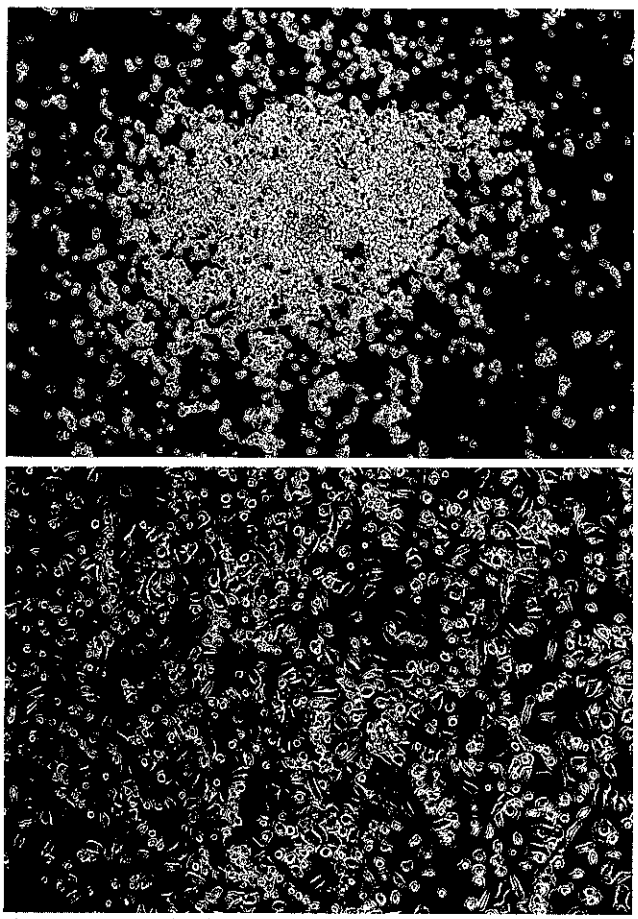

Fig. 7. Stromal-cell dependent growth of cultured tumor cells. a, tMK-2 tumor cells metastasized to the spleen were used for this experiment. *In vitro* growth of tMK-2 cells by attaching to splenic stromal cells. Photographs were taken after one month in culture. b, After detachment of tMK-2 tumor cells by vigorous shaking from the adherent splenic stromal cells of the nude mouse.

tMK-2 tumor-bearing mouse, slowly growing lymphoid tumor cells could be obtained. Several passages of the cells in culture were carried out over more than a year. In Fig. 7-a, round tumor cells which only grow attached to the spindle-shaped stromal cells (Fig. 7-b) of the spleen of nude mice are shown. The growth properties of the liver tumor cells in the presence of the liver stromal cells were essentially similar. When we separated these tumor cells from the stromal cells and cultured them in the absence of the stromal cells, these tumor cells died within ten days. These cells thus were growing in a stromal-cell dependent manner.

Phenotype analysis of tumor cells *In vivo*-maintained lymphoma cells were freshly obtained from the liver of nude mice and isolated by Ficoll-Hypaque density centrifugation. *In vitro* cultured lymphoma cells were incubated for 1 h, then non-adherent cells to the plastic culture flask were collected. The cells were stained with FITC-labeled anti-mouse anti-CD2, CD3, CD4, CD8 and Mac 1 MoAbs or PE-labeled anti-mouse TcRα/β, H2K^d, or Ig (κ) MoAbs.

Table III. Phenotype Analysis and Organs Preferentially Affected by Developed Lymphomas

Lymphoma	Phenotype[a] (liver)		Preferentially affected organs in addition to liver and spleen[b]
	in vivo	*in vitro*	
tMK-2	T[c]	T	—
tHK-1	T	T	lymph nodes of extremity
tYK-1	T	T	lymph nodes of extremity
tYK-2	T	T	uterus
tTY-1	T	T	uterus, mesenteric lymph nodes

a) Phenotyping was performed by FACS analysis. T-Cell phenotype means that the lymphoma cells showed more than 95% reactivity with anti-CD3 and anti-TcR monoclonal antibodies.
b) Upon autopsy, the organs macroscopically affected by lymphoma cells were observed. Details are described in "Materials and Methods."
c) Hepatic lymphocytes were prepared by Ficoll-Isopaque density (1.090) gradient centrifugation.

The results are summarized in Table III. All of the developed lymphomas showed exclusively T-cell phenotype and no lymphoma cells showed B or null cell phenotype. Organs macroscopically affected during *in vivo* maintenance of these lymphomas, in addition to the liver and spleen, are also shown.

In Fig. 8, more detailed phenotypes of tMK-2 and tYK-1 cells are shown. Both of these lymphoma cell lines reacted strongly with anti-CD2 (99.0% and 97.5%, not shown in the figure), CD3 and TcRα/β MoAbs, but did not react with anti-CD4, anti-CD8, anti-Mac 1 or surface Ig (κ) MoAbs, indicating that these tumor cells belong to the early T-cell lineage but not to the monocyte, B cell or null cell lineage. These tumor cells reacted weakly with anti-mouse H2K^d histocompatibility antigen MoAb (10.1% and 8.1%). Other lymphoma cells, tHK-1, tYK-2 and tTY-1 showed essentially the same results (data not shown in Fig. 8).

DISCUSSION

This is the first report to present a clear characterization of murine T-cell lymphoma developed in antigenically stimulated BALB/c nude mice. One of the most important observations is the induction of T-cell phenotype-bearing lymphomas in nude mice. Whether or not T lymphocytes can differentiate without passing through

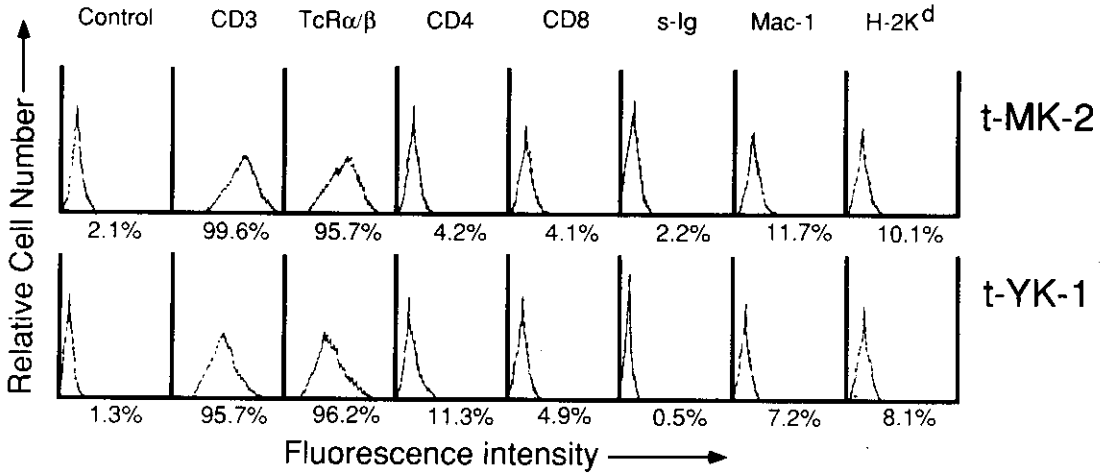

Fig. 8. Flow cytometry of tumor cells. Freshly isolated tMK-2 and tYK-1 tumor cells were subjected to phenotype analysis by flow cytometry after staining with FITC- or PE-labeled MoAbs. Positive expression of both the CD3 and TcRα/β antigen can be seen. Faint expression of MHC-class I molecule H-2K^d should be noted.

the thymus is not clear, and many points remain to be elucidated. We have shown here that the tumors developed in the liver express typical characteristics of T lymphocytes of extrathymic origin, such as TcRα/β-positive and CD4/8 double-negative phenotypes. To our knowledge, this is the first cell line with the characteristics of extrathymic T-cells in the liver.

Baird *et al.*[9] found that antigenically induced lymphoma cells are a mixture of different phenotypes in which the dominant phenotype is B/null cells and the T-cell phenotype is a minority. They claimed that, even under *in vitro* culture conditions, there is a mechanism for the selection of lineage-specific growth and again the T-cell phenotype is a minority.[9] However, under our experimental conditions, the developed lymphomas showed a hundred percent T-cell phenotype both *in vivo* and *in vitro*, without exception. In addition, as shown in Fig. 8, the tMK-2 and tYK-1 tumor cells express mouse CD2, CD3 and mouse TcRα/β antigens but do not express CD4 or CD8 antigens. These cells thus belong to an early T-cell lineage. Concerning the low level expression of H-2K^d, one possible explanation is as follows. From circumstantial evidence, the extrathymically developed T-cells in the liver are considered to be evolutionarily classified between natural killer (NK) cells and intrathymically differentiated T-cells. This would be why extrathymic T-cells express a historical and monomorphic H-2, such as TLA, and not polymorphic modern H-2.

Recently, this new type of T-cell has been reported to be classified as a prototype T-cell, differentiated without thymic selection, preferentially in the homing organs such as lymph nodes or liver.[15] They show morphologically an intermediate type between thymus-derived T-cells and NK cells. These newly found cells thus might serve as prototype cells to analyze the historical development of immune systems. We speculate that these extrathymically developed T-cells play an important role in the immune-surveillance of host defense mechanisms against atypical cells generated *in vivo*, in conditions such as cancer development, auto-immune diseases, aging, graft-versus-host (GVH) reactions, pregnancy and parasitism, where the thymus becomes atrophic.[16] On the other hand, thymus-derived T-cells, which differentiate after negative selection to eliminate auto-reactive clones,[17] play a role in protection from foreign antigens. For the analysis of such phenomena, the establishment of cell lines is considered essential.

The incidence of spontaneous tumor development in long-lived nude mice is reported to be relatively low. For instance, Outzen *et al.*[18] found spontaneous development of lymphoma in only 2% of BALB/c nude mice (200 mice, life span from 30 to more than 400 days). Tamaoki[19] also reported lymphoma development in only 1.9% of 309 nude mice with a 3–18 month life span. Spontaneous lymphomas of B-cell origin occur infrequently, at a rate of 1/250 to 1/500 and at an age of 12–24 months.[10]

Transformation of host stromal cells by xenografts has been reported.[3-8] The host fibroblast cells can be transformed *in vivo* or *in vitro*, resulting in the formation of fibrosarcomas.[4-8] The incidence of the transformation of effector cells, such as lymphocytes, by inoculation of tumor cells still remains unclear. For instance, frequent

induction of lymphoma by human tumor xenografts as well as chronic pinworm infestation has been reported.[9] Subsequent to the above report, however, only a few case reports have appeared, such as B-cell lymphoma induction in athymic NIH Swiss nu/nu mice by inoculation with NIH 3T3 cells transfected with a human oncogene, hh_cM,[10] and the development of lymph-sarcoma lines in BALB/c mice during subcutaneous passage of Colon 26 adenocarcinoma.[11]

In our study, chromosome analysis and DNA pattern analysis by electrophoresis revealed that all of the developed tumors and cultured cell lines were of mouse origin. Tumors developed in five out of five independent surgical specimens (100%) or seven out of twelve inoculated mice (58%) and these tumors developed from 2.5 to 9 months after inoculation of surgical specimens. No tumor development was seen in fourteen nude mice comprising ten nude mice inoculated with five different esophageal cancer cell lines and 4 nude mice inoculated with two breast cancer specimens of the non-inflammatory type for periods of up to twelve months and ten months, respectively. Our finding of a high frequency rate of tumor development less than one year after the inoculation of surgical specimens of inflammatory breast cancer patients apparently resembles that of Baird *et al.*[9] However, our results are clearly different from those of Baird *et al.*,[9] since we obtained a frequent lymphoma induction (58%) by inflammatory breast cancer as compared with 0% lymphoma induction in 14 control tumor-inoculated mice, while they reported a frequent lymphoma induction (63%) by human tumor xenografting and a frequent lymphoma induction (56%) by chronic pinworm infestation.

During a period of 20 years since the discovery of the athymic mouse, our animal facility, which is operated under specific pathogen-free (SPF) conditions, has examined more than 5000 implantations of human tumor cell lines and surgical specimens from a variety of cancer tissues, including stomach, pancreas, liver, colon, breast, etc., in nude mice. The incidence of tumor take is about 30% and this percentage is consistent with reported values. Our animal facility systematically looks at the implanted mice for a period of 4 months and the mice are killed when the tumor implantation is judged unsuccessful. Under these conditions, we never found lymphoma induction in approximately 3500 nude mice. We think, therefore, that some unknown factors including environmental conditions are deeply involved in the induction of the murine lymphomas after xenografting.

Our results also strongly suggest that the tumor development was not spontaneous, but was induced by the human cancer xenografting. The mechanism of malignant transformation of host cells might depend on the activation of oncogenes,[20] the presence of a host virus,[5,9,21] cell fusion[22,23] or growth factors.[24,25] Another possibility is horizontal transfer of a human virus such as Epstein-Barr virus (EBV), human papilloma virus (HPV) or an unknown pathogenic retrovirus or non pathogenic virus with considerable homology to murine mammary tumor virus (MMTV).[26,27] The MMTV-like virus is reported to be present in some human breast cancer cells.[28] The mechanism of the induction of T-cell lymphoma of murine origin by inoculation of inflammatory breast cancer remains to be elucidated.

During the maintenance of tumor cells *in vivo* by subcutaneous inoculation of tumor cells, we found an obviously inflammatory process, including skin erythema, around the growing tumors. This inflammatory process, whether generated by direct or indirect mechanisms, could be involved in the induction of lymphoma. The pattern of dissemination to the spleen and liver has often been described for tumors of the hematopoietic systems.[29–33] Our cases are consistent with this pattern of tumor distribution. It was also found that the tumors developed in the spleen and liver in nude mice inoculated with the surgical specimens of donor patient MK, but in the mice of subsequent donor patients HK, YK and TY, tumors simultaneously developed in the axillar, inguinal, submandibular and mesenteric/intestinal lymph nodes and uterus in addition to the spleen and liver. These tumor-bearing mice might serve as good modes to analyze factors responsible for organ-specific metastasis and growth of tumor cells.

We transferred tMK-2 tumor cells into *in vitro* culture. Interestingly, these tumor cells grow only in the presence of stromal cells from the metastasized visceral organs, spleen or liver. The transfer of these tumor cells in suspension form to other flasks in the absence of stromal cells resulted in the death of the tumor cells within ten days.

Our cell lines, the first of their type to our knowledge, thus might serve as a tool for studying the role of T-cells with TcRα/β^+, $CD4^-/8^-$ phenotype as well as for studying the mechanisms of *in vivo* transformation of murine cells by xenografted human inflammatory breast cancer.

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