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Establishment of Transplantable Tumor Lines and *in vitro* Cell Lines of Malignant Thymoma Developed in a BUF/Mna Rat

Jin Lu,[1,6] Yoshihisa Sakai,[1] Worawidh Wajjawalku,[1] Ken-ichi Isobe,[2] Mitsuhiro Saito,[1] Hiroyuki Amo,[3] Akinori Kojima,[4,7] Kazuhiko R. Utsumi,[5] Masahide Takahashi,[1] Hiroshi Hiai[4,8] and Mutsushi Matsuyama[1,9]

Departments of [1]Pathology and [2]Immunology, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466, [3]Aichi Women's College, 57 Takenoyama, Iwasaki, Nisshin-cho, Aichi-gun, Aichi-ken 470-01, and Laboratories of [4]Experimental Pathology and [5]Ultrastructure Research, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya 464

A spontaneous malignant thymoma was found in an 18-month-old female BUF/Mna rat and serially transplanted subcutaneously in both syngeneic BUF/Mna rats (designated as MTH-R) and KSN nude mice (MTH-NM) for more than 5 years. Both tumors shared the histological appearance of sarcomatoid carcinoma as seen in the original tumor. However, MTH-NM grew faster than MTH-R in the respective hosts. The MTH-NM grew in both KSN-nude mice and BUF/Mna-*rnu/rnu* rats but not in BUF/Mna rats, the host of the original tumor. Three continuous tissue culture cell lines (MTHC-1, MTHC-2 and MTHC-3) were established from the MTH-NM tumors at the 2nd, 15th and 17th transplantation generations, respectively. The MTH-NM tumors and latter two tissue culture cell lines carried one or more mouse chromosomes, probably acquired by cell fusion with mouse cells during passages *in vivo*. The presence of the mouse chromosomes was confirmed by the presence of mouse DNA and of antibodies to the MTHC-2 and MTHC-3 cells in the sera of BUF/Mna rats transplanted with MTH-NM.

Key words: Transplantable malignant thymoma line — BUF/Mna rat — *In vitro* cell line — Growth acceleration — Hybridized cell

We have reported that BUF/Mna rats are genetically predisposed to spontaneous development of thymoma at a high incidence.[1-4] As in human thymomas, the essential component of the BUF/Mna thymomas is epithelial cells, while the thymomas also contain a variable number of non-neoplastic lymphoid cells. Most of the thymomas show lymphocytic or mixed-type histology but a few of them are of epithelial type. Most thymomas are biologically benign, e.g., growing slowly and expansively, and remote metastasis is rarely found. Transplantation of thymomas has often been attempted, but with success only in newborn recipients. Recently a malignant thymoma was found in an aged female BUF/Mna rat. From this tumor, we were able to generate transplantable tumor lines as well as tissue culture cell lines. Chromosome analyses revealed that these tissue culture cell lines retained mouse chromosomes which probably resulted from hybridization *in vivo*.[5] This paper describes some properties of the malignant thymoma in a BUF/Mna rat, and of its sublines maintained both *in vivo* and *in vitro*.

MATERIALS AND METHODS

Animals The origin of BUF/Mna strain rats was described previously.[3,4] They were maintained in our laboratory by strict brother-sister mating. Rat nude gene (*rnu*) was introduced from a female N:NIH-*rnu*/+ rat to rats of the BUF/Mna strain in our laboratory by serial backcrossings and BUF/Mna-*rnu/rnu* rats of both sexes were used at the 17th backcross generation. Six-week-old female KSN nude mice were supplied from the Institute for Animal Research, Nagoya University School of Medicine.

Microscopic and submicroscopic examinations For all tumors, routine histological examination was carried out with hematoxylin-eosin-stained paraffin sections. Immunoperoxidase stainings of paraffin-embedded tumor tissues were performed by the technique of Sternberger.[6] Sections were first incubated with 1:50 diluted rabbit polyclonal anti-human keratin antiserum (DAKO, Denmark), washed, and then incubated with 1:2000 peroxidase-conjugated goat anti-rabbit IgG (DAKO).

For electron microscopy, small pieces of minced tumor were fixed in 2.5% glutaraldehyde plus 2% para-

[6] Present address: Department of Pathology, Sasaki Institute, 2-2 Kanda-Surugadai, Chiyoda-ku, Tokyo 101.
[7] Present address: Environmental Health Department, Nagoya City Public Health Research Institute, 1-11 Hagiyama-cho, Mizuho-ku, Nagoya 467.
[8] Present address: First Department of Pathology, Kyoto University School of Medicine, Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606.
[9] To whom all correspondence and reprint requests should be addressed.

formaldehyde in 0.05 *M* phosphate buffer, post-fixed in 1% osmium tetroxide, dehydrated in graded ethanol, and embedded in Epon 812. For electron microscopy of cultured cells, 5×10^4 cells were grown in two 25-cm^2 Falcon tissue culture flasks for 4 days. The cells were harvested by scraping monolayers with a rubber policeman and spun down at 800 rpm for 5 min. The pellets were processed as described above. Ultrathin sections were cut with an LKB ultratome (LKB, Bromma, Sweden), stained with uranyl acetate and lead citrate, and examined in a Hitachi H-600 electron microscope.

Transplantation In the original tumor, there were macroscopically distinct white hard and pinkish soft parts. Small blocks of each part were separately transplanted into the subcutaneous region of either side of the axilla of young BUF/Mna rats and KSN nude mice. The transplants from the hard part of the tumor grew in all of the recipients, but those from the soft part did not grow in any recipient.

Measurement of *in vivo* growth rates of MTH-R and MTH-NM About 90 mg of MTH-R and MTH-NM was subcutaneously transplanted in the right axillary region of 29 BUF/Mna rats and 20 KSN nude mice, respectively. Every 2 weeks, 4 or 5 rats were killed and the subcutaneous tumors were weighed. Four mice were also killed weekly and the tumors were weighed. The measurement was discontinued at the 12th and 5th week after transplantation in MTH-R and MTH-NM, respectively, because of ulcer formation.

Cell culture Tumors passaged in KSN nude mice were dissected and minced with scissors in Hanks' balanced salt solution (HBSS) containing penicillin (100 U/ml) and streptomycin (100 μg/ml). After one wash in HBSS, the fragments were placed in 6-cm plastic dishes (Falcon Plastics, Los Angeles, CA) with 3.5 ml of Dulbecco's modified Eagle's medium (DMEM, GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum. Culture was carried out at 37°C in a CO_2 incubator with 5% CO_2 and 95% fully humidified air. The medium was changed every week. Cells were passaged by digestion with 0.1% trypsin and 0.05% EDTA for 5 min at 37°C when they reached confluence. From the 2nd passage, cells were maintained in plastic flasks.

***In vitro* cell growth** The growth rate of cultured thymoma cell lines in the log phase was measured. To standardize culture conditions, free cell suspension was carefully prepared by trypsin-EDTA digestion of a log-phase monolayer culture and 6-cm plastic dishes were each inoculated with $1–2\times10^5$ free cells in 3.5 ml of growth medium. Every day, starting the day after initiation of the culture, cells were harvested by trypsinization and the cell numbers were counted with a hemocytometer. Each measurement is given as an average of cell counts of more than 4 dishes.

Chromosome analysis Chromosome preparations were made by the ordinary air-drying method. G-Banded chromosomes were obtained by trypsin treatment and stained with Giemsa's solution.[7] Rat chromosomes were identified according to Levan's nomenclature,[8] and mouse chromosomes according to the standard karyotype.[9]

Immunofluorescence and laser flow cytometry MTHC-1, MTHC-2 and MTHC-3 cells were reacted with sera obtained from male BUF/Mna rats transplanted with MTH-NM or MTH-R as the first antibody for 30 min in 4°C. After washing three times, the cells were suspended in the medium containing the second antibody (FITC-conjugated anti-rat IgG goat antiserum, Tago Inc., Berlingame, LA) and incubated for 30 min in 4°C. After washing three times, the cells were resuspended in medium and analyzed on an EPICS profile fluorescence-activated cell sorter (Coulter Corporation, FL). The fluorescence intensity was recorded for 5000 cells in a diagram of logarithmic fluorescence intensity (LFL: log fluorescence) versus cell number (on the y axis).

RESULTS

Original tumor A thumb-sized tumor with lobulations was found incidentally in the upper mediastinum of an 18-month-old female BUF/Mna rat that died on March 11, 1987. The tumor compressed the bilateral lungs and heart. Macroscopically it consisted of a main white hard part and continuing pinkish soft part at the lower left corner. Several small bean-sized white hard deposits were also disseminated on the pleura of the bilateral chest cavities. Histologically, the white hard part of the main tumor and pleural deposits was com-

Fig. 1. Junctional area between the white hard and pinkish soft parts of the original tumor, showing a sarcomatoid carcinoma pattern on the left and mixed thymoma on the right. H-E. ×170.

posed of atypical fusiform cells with vesicular nuclei and eosinophilic cytoplasm (Fig. 1). A few tumor cells showed a moderate reaction to anti-keratin antiserum. Sub-microscopically, many desmosome-like attachments were found on the cell membrane, and a few bundles of fine filaments were observed in the cytoplasm. Therfore, the white hard part of the tumor was diagnosed as sarcomatoid thymic carcinoma[10, 11] and the pleural deposits were diagnosed as its metastases. The pinkish soft part was histologically of typical mixed-type thymoma[1] (Fig. 1).

Transplantable malignant thymoma line passaged in BUF/Mna rats (MTH-R) A transplantable thymoma line MTH-R was established by inoculating tissue fragments from the white hard part of the original tumor into the right axillary region of BUF/Mna rats and serially passaging the tumors for 15 transplantation generations during 5 years. The MTH-R tumors invariably grew as a hard round mass, sometimes invading the skin and killing the recipients within about 6 months. They showed a sarcomatoid carcinoma pattern with fibrosis (Fig. 2) and hyalinization in the central area. The doubling time *in vivo* was about 187 h.

Transplantable malignant thymoma line passaged in KSN nude mice (MTH-NM) MTH-NM was serially transplanted in KSN nude mice in the same way as MTH-R in BUF/Mna rats and reached the 31st transplantation generation. The MTH-NM tumor grew faster than MTH-R with a doubling time of about 142 h. It formed a hard round mass, invading the surrounding tissues such as the skin, muscles and bones, and killed the recipients in about 3 months. Histologically, the tumors were slightly more cellular than MTH-R and less fibrous (Fig. 3). They contained a large area of central necrosis. Submicroscopically, a few bundles of fine filaments were observed in the cytoplasm of a few tumor cells (Fig. 4), as in the original tumor.

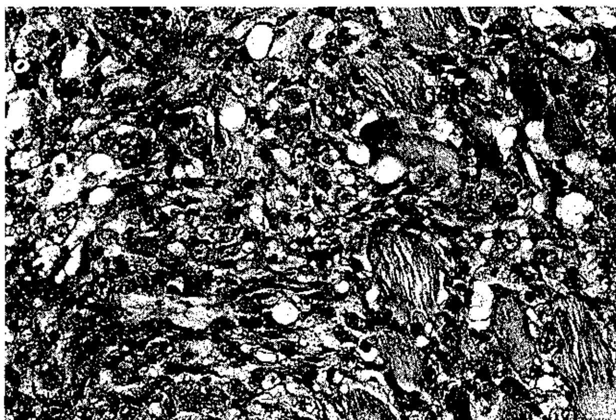

Fig. 2. An area of MTH-R, at the 14th transplanted generation, infiltrating chest wall muscles. The tumor consists of fusiform cells with vesicular nuclei. H-E. ×280.

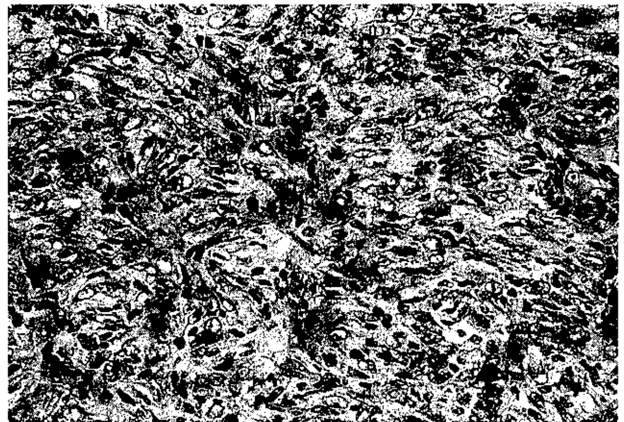

Fig. 3. An area of MTH-NM, at the 25th transplanted generation. The tumor cells have vesicular nuclei. H-E. ×280.

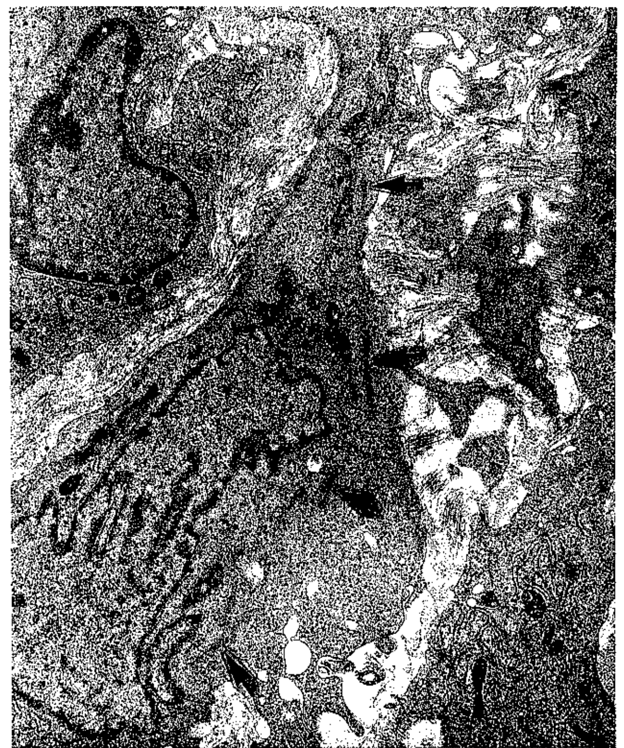

Fig. 4. An electron micrograph of cells of MTH-NM, 16th transplanted generation. Bundles of fine filaments (arrows) are scattered in the cytoplasm. ×9,000.

When the MTH-NM tumor was transplanted in 5 BUF/Mna-*rnu/rnu* nude rats, it grew as rapidly as in the KSN nude mice. However, the transplants from MTH-NM at the 28th transplantation generation were rejected in all of 6 BUF/Mna rats, the original host of the tumor.

Establishment of cultured cell lines and their growth characteristics Three cell lines, MTHC-1, MTHC-2 and

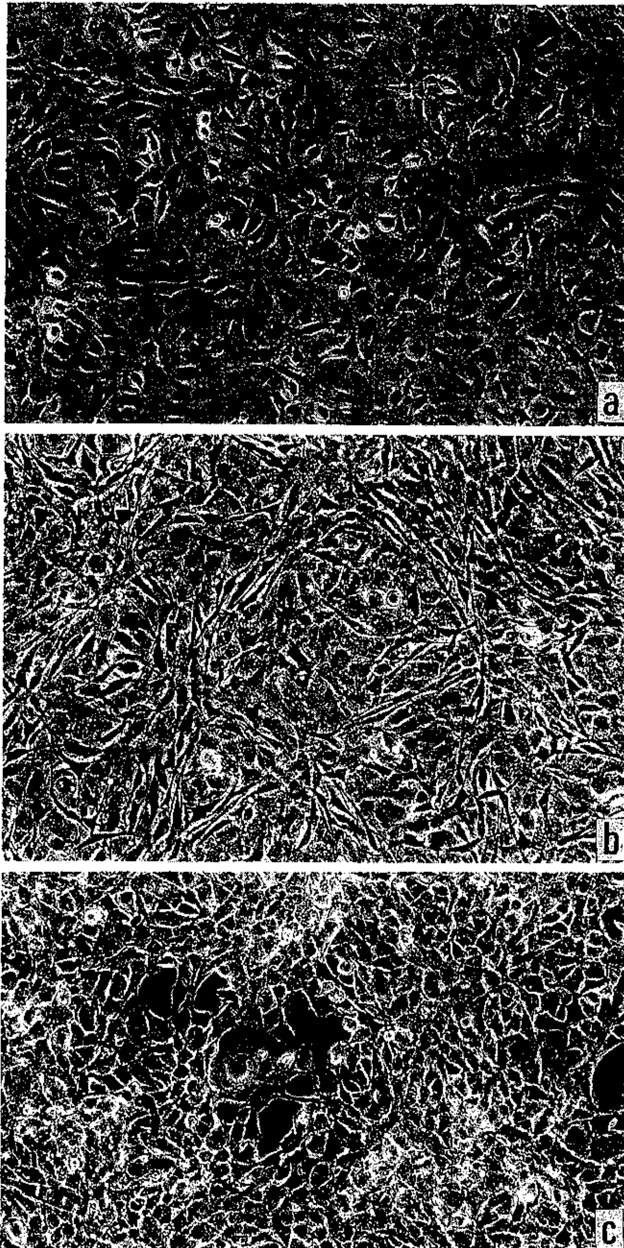

Fig. 5. Phase-contrast micrographs of the MTHC-1, passage >150 (a), MTHC-2, passage 24 (b), and MTHC-3 cell lines, passage 60 (c).

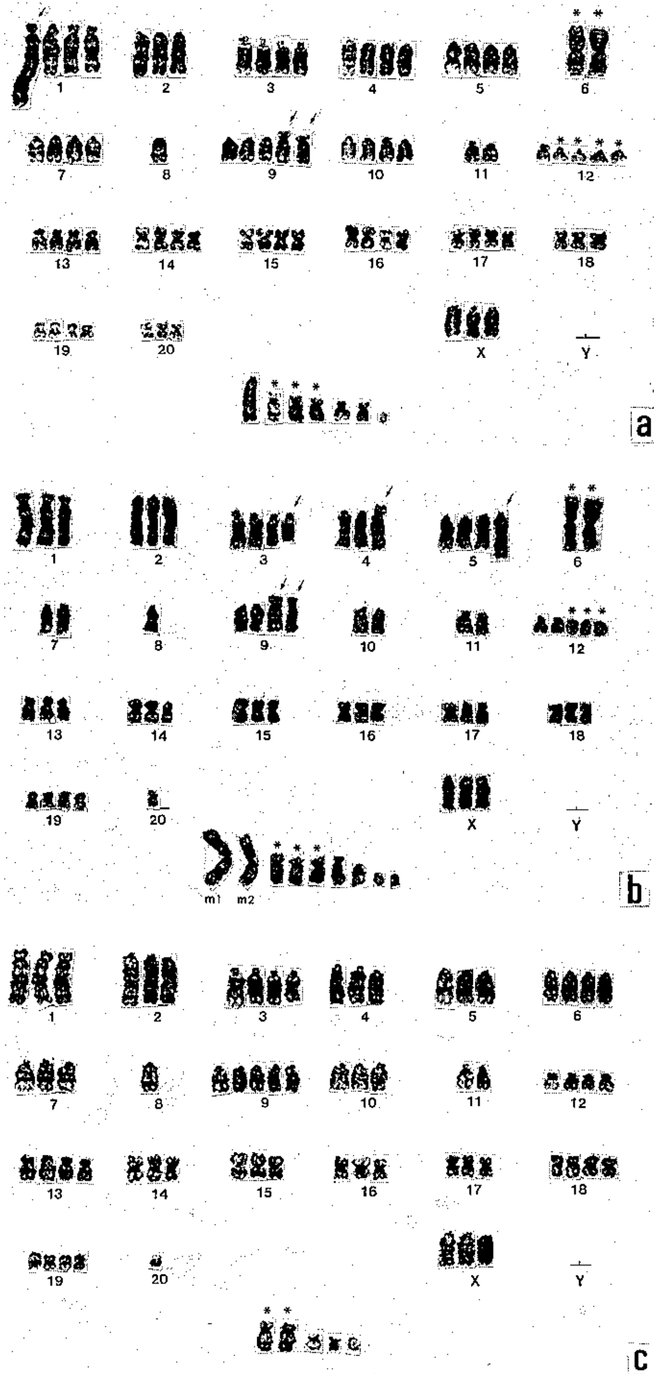

Fig. 6. Karyotypes of cells of MTH-R, at the 7th transplanted generation (a), MTHC-2, at the 10th passage (b), and MTHC-1, at >120th passage (c). Unidentified chromosomes are arranged in the bottom line. Arrows: derivative chromosomes. *: common markers. Monosomy of chromosome 8 is common to all cell lines. Deletions of 12p and t(6q6q) are commonly found in all cell lines except the MTHC-1 line. ml: probably a Robertsonian fusion of mouse chromosome 5s. m2: probably a Robertsonian fusion of mouse chromosomes 15 and 17.

Table I. Chromosome Analysis of Transplantable Malignant Thymoma of Rat (MTH-R) and *in vitro* Cell Lines (MTHC-1, MTHC-2 and MTHC-3) Derived from Transplantable Malignant Thymoma in Nude Mice (MTH-NM)

	MTH-R, G 7[a] after short term culture	MTHC-1 cell line >P 120[a]	MTHC-2 cell line P 1[a]	MTHC-2 cell line P 10[a]	MTHC-3 cell line P 2[a]
No. of cells examined	14	10	21	31	18
Modal number	81–83	69–71	74	73	70
rat t(6q6q)×2	+	−	+	+	+
rat 8 monosomy	+	+	+	+	+
rat $9p^+$	+	−	+	+	+
rat $12p^-$	+	−	+	+	+
rat marker	+	+	+	+	+
mouse 5	−	−	2 (10)[b]	−	4 (22)
mouse t(5q5q)	−	−	14 (67)	29 (94)	−
mouse t(5q7q)	−	−	1 (5)	−	10 (56)
mouse t(15q15q)	−	−	1 (5)	−	−
mouse t(15q17q)	−	−	2 (10)	12 (39)	17 (94)
Cells with mouse chromosomes	0 (0)	0 (0)	17 (81)	29 (94)	17 (94)

a) G: generation in tumor transplantation. P: passages in culture.
b) Number in parenthesis is the percentage.

MTHC-3, were established from MTH-NM at the 2nd, 15th and 17th transplantation generations, respectively. They were cultured for more than 40 weeks. Under phase-contrast microscopy, the MTHC-1 cells were polygonal in shape and proliferated as a sheet (Fig. 5a). The MTHC-2 and MTHC-3 cells showed a network structure, consisting of 2 different shapes of cells, polygonal cells that contained many lipid vacuoles and elongated cells (Fig. 5b, c). A few giant cells containg a single large nucleus or multiple nuclei were seen sporadically in MTHC-1 and MTHC-3. Submicroscopically, a few cells of these cell lines had fine filaments and desmosome-like apparatuses, as in the white hard part of the original tumor. The population doubling times of the MTHC-1 (at passage >150), MTHC-2 (at passage 41) and MTHC-3 (at passage 41) cell lines were estimated to be 20.3, 23.7 and 27.1 h, respectively, during the exponential phase of growth.

All of the three cell lines retained tumorigenicity in nude mice with virtually 100% takes. However, the MTHC-2 and MTHC-3 cells, which were found to carry mouse chromosomes as described later, did not produce any tumor in any of 9 BUF/Mna rats, whereas the MTHC-1 cells produced tumors in all of 6 BUF/Mna rats.

Chromosome analysis of MTH-R cells and *in vitro* cells of MTHC-1, MTHC-2 and MTHC-3 These tumor and cell lines, either *in vivo* or *in vitro,* were hypotetraploid with a modal number between 69 and 83 (Table I). Monosomy of chromosome 8 and unidentified chromosomes (with * arranged in the bottom line in each karyotype) were commonly found in all cell lines (Fig. 6a, b, c). Two pairs of t(6q6q) and $12p^-$ chromosomes were common to all cell lines but MTHC-1, in which chromosomes 6 and 12 showed normal tetrasomy (Fig. 6c). Interestingly, the MTHC-2 cells had the mouse chromosome(s); No. 5, 7, 15 and 17. The single chromosome 5, t(5q7q) and t(15q15q) were rare at the 1st passage, but t(5q5q) and t(15q17q) (ml and m2), respectively (Fig. 6b), were maintained in the early and late passages. The mouse chromosomes were also found in MTHC-3 cells: single chromosome 5, t(5q7q) and t(15q17q) (Table I).

^{32}P-Labeled total mouse DNA hybridized to mouse repetitive sequences in DNAs of MTHC-2 and MTHC-3 cells, but not in DNA of MTHC-1 cells (data not shown).

Presence of antibody against mouse antigens in sera of BUF/Mna rats challenged with MTH-NM In order to show the presence of mouse cell surface antigens on MTHC-2 or MTHC-3 cell lines, sera taken from BUF/Mna rats at 10th day following the transplantation of MTH-NM or MTH-R were used for immunofluorescence examination. Ten-times-diluted sera were reacted with MTHC-1, MTHC-2 and MTHC-3, and mixed with FITC-conjugated anti-rat IgG. Peaks of the fluorescence intensities were the same in MTHC-1 cells reacted with sera obtained from rats transplanted with MTH-NM and MTH-R (Fig. 7a). However, the sera from MTH-NM gave higher peaks of fluorescence intensities in MTHC-2 and MTHC-3 cells, which have mouse chromosomes, than the sera from MTH-R (Fig. 7b, c).

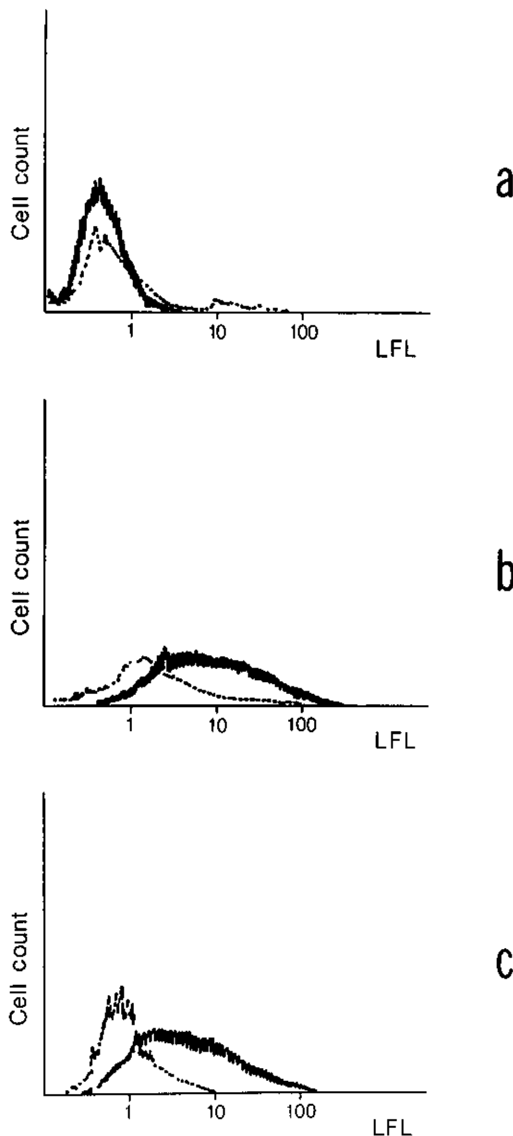

Fig. 7. Distributions of the immunofluorescence intensities of MTHC-1 (a), MTHC-2 (b) and MTHC-3 cells (c), when reacted with sera obtained from BUF/Mna rats challenged with MTH-NM (solid line) and MTH-R (dotted line).

DISCUSSION

We have established transplantable thymoma lines and tissue culture lines from a malignant thymoma of a BUF/Mna rat. To our knowledge, no transplantable thymoma line has previously been reported. The establishment of thymoma cell lines *in vitro* is difficult because of their low propagation ability and contamination by rapidly growing fibroblasts. Only 8 cell lines from human thymomas[12] and 7 clonal cell lines from a rat thymoma[13] have hitherto been reported. So far, no cell line has been established from a malignant thymoma that is transplantable to nude mice. The present study reports successful establishment of malignant thymoma lines and their *in vitro* cell lines.

The tumors passaged in syngeneic BUF/Mna rats and in KSN nude mice showed similar histological appearance to the original tumor. However, MTH-NM, maintained in KSN nude mice, grew faster than MTH-R maintained in BUF/Mna rats, with a doubling time of about three-fourths of that of the latter. The mechanism of the faster growth is not clear. Host species difference in the growth rates is unlikely, since the MTH-NM tumors, when transplanted in BUF/Mna-*rnu/rnu* nude rats, grew as fast as in KSN nude mice. In contrast, growth of MTH-R is not accelerated in BUF/Mna-*rnu/rnu* nude rats. Therefore, the most likely explanation is that cells well adapted to the nude mouse host environment have been selected in the MTH-NM tumor during the many passages in nude mice.

Chromosome analysis of the thymoma lines indicated that they are hypotetraploid and that the *in vitro* cells derived from the MTH-NM contain the mouse chromosomes 5, 7, 15 and/or 17. These observations indicate that MTH-NM is a spontaneous hybrid formed *in vivo* between rat malignant thymoma cells and mouse normal cells, though most of the mouse chromosomes had been lost. Spontaneous *in vivo* hybridization of cells is a rare phenomenon. Our previous study, however, showed that epithelial cells derived from BUF/Mna rat thymomas were prone to fuse with each other or with macrophages.[14] It has repeatedly been reported that hybridized cells have a growth advantage.[15-18] The mouse chromosomes found in MTH-NM may have gene(s) that accelerate the growth of the tumor. The rejection of MTH-NM by BUF/Mna rats may well be explained by the presence of the mouse surface antigens and of mouse chromosome 17, which carries major histocompatibility complex genes.[19]

MTH-R and MTH-NM, and *in vitro* cell lines, MTHC-1, MTHC-2 and MTHC-3, may be useful not only for studies on the mechanism of development and progression of thymomas but also for evaluation of candidate antitumor drugs against malignant thymomas.

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