Establishment of MN-Lymphosarcoma as a Permanent Cell Strain in Tissue Culture

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SYNOPSIS

Three tissue culture strains were established from MN-lymphosarcoma cells originally carried intraperitoneally in NA_2 female mice. They have been maintained by serial subcultivation for more than 16 months. There were no apparent differences except for cell size in morphological features among the 3 strains. When inoculated into mice intermittently after prolonged maintenance *in vitro*, strain No. 1 and No. 3 cells induced ascites tumor formation and a mortality of 80～100%. Strain No. 2 cells seemed to have lost their transplantability. When the cultured strain No. 1 cells were carried serially by mouse-to-mouse passage, the mean survival time, which was longer in the first passage, was shortened within a few generations to that of the original mouse-to-mouse passage. This result might be discussed on the hypothesis of a gradual transformation of cells for adaptation to growth in altered environment.

INTRODUCTION

The use of murine ascites tumor cells as an experimental tool for cancer research has proved favorable and a considerable amount of literature on this subject has been published.

Under *in vitro* conditions, cells can be grown with elimination of many complicating factors that exist in the whole body, providing more profitable material for investigation because of relatively easy management with the use of semi-synthetic media. In recent times, attempts have been made to establish and maintain animal ascites tumor in culture and those which have succeeded in long-term culture were the AH-130 rat ascites hepatoma,[31] 6C3HED mouse lymphosarcoma,[11] TA_3mammary carcinoma,[12] and Krebs-2 and Ehrlich ascites carcinoma.[1, 6, 7, 10, 11, 30]

In our laboratory, the ascites form of MN-lymphosarcoma has been employed for studies on correlation between steroidal hormones and tumor cells and this made it

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necessary to adapt these cells to growth *in vito*. Fundamental conditions suitable for the cultivation of the cell were examined in a previous work.[21]

The present paper describes the processes of cultivation *in vitro* leading to the establishment of 3 strains and the induction of ascites tumors in mice by the inoculation of these strains.

MATERIALS AND METHODS

Tumor Cell The T-strain of MN-lymphosarcoma (hereinafter abbreviated as MN/T) was produced by Mori and his associates in an inbred NA_2 female mouse by a long-term administration of hexestrol, a synthetic estrogen, as described in detail in the preceding paper.[21] MN/T was converted to an ascites form and has been carried by weekly intraperitoneal passage in NA_2 female mice. It was obtained from the Department of Pathology of this medical school in 1960. Since the establishment of MN/T, it has neither been frozen nor cloned. Prior to the present experiments MN/T underwent animal passage for 10 months in our laboratory.

Culture Medium Hanks balanced salt solution containing 0.5% lactalbumin hydrolysate (Enzymatic, Nutritional Biochemicals Corp., U.S.A.) and 0.1% yeast extract (Difco) supplemented with 20% bovine serum, was used in the beginning. Since the optimal concentration of bovine serum was determined in the preceding work,[21] the medium was modified to contain 40% bovine serum, 0.4% lactalbumin hydrolysate, and 0.08% yeast extract in the Hanks saline. Penicillin and dihydrostreptomycin were added to the medium in a final concentration of 30 U/ml and 30 γ/ml, respectively.

Immediately after the medium was prepared, it was sterilized by passing through a Seitz filter and adjusted to pH 7.6. Bovine serum was inactivated by heating at 56° for 30 minutes before filtration.

Culture Method Short test tubes (15 × 110 mm), roller tubes (15 × 150 mm), and dilution bottles (50 × 50 × 100 mm) were used as the culture vessel. They were sealed with double stoppers. More recently, only short test tubes have been employed.

From the result shown in the previous paper,[21] a static culture method was used throughout this experiment. Culture vessels were incubated stationarily at an angle of 5° from the horizontal, at a temperature of 37°.

Primary Culture Ascitic fluid was withdrawn aseptically by means of a glass capillary from mice for the primary culture of each of the 3 strains.

1) Strain No. 1: Tumor cells were obtained, on September 20, 1961, from the peritoneal cavity of a mouse 6 days after routine tumor inoculation. Two drops of this ascites were mixed with 30 ml of culture medium supplemented with 20% bovine serum in a dilution bottle and cells in the mixture were counted with a hemocytometer. Two-ml aliquot containing approximately 10^5 cells was divided into 10 roller

tubes and these were incubated for 10 days. At an interval of 48 hours, as the pH became acid, cultured vessels were set in a standing position and kept for 1 hour. One-half volume of the old medium was removed by pipetting off the cell-free supernatent and replaced by a fresh medium.

2) Strain No. 2: On September 26, 1961, ascites was acquired from a mouse inoculated 7 days previosly. All procedures for cultivation except the composition of the medium were the same as that used for strain No. 1. The culture medium consisted of 40% bovine serum, 0.4% lactalbumin hydrolysate, 0.08% yeast extract, and Hanks saline. Ten roller tubes were incubated at 37° for 8 days.

3) Strain No. 3: Ascitic fluid was derived, on Octoder 7, 1961, from a mouse 8 days after intraperitoneal inoculation of the preceding ascites. The fluid was mixed in a dilution bottle with 30 ml of the medium similar to the one used for strain No. 2 and the number of cell nuclei was 10^5/ml. The dilution bottle was incubated in a stationary position for 30 days. The medium was renewed at 3- to 4-day intervals depending on pH of the medium.

Subculture MN/T cells grew without adhering tightly to the glass surface but floating at the bottom during cultivation and showing a tendency to form groups of several to more than ten cells.[21] Cells were dispersed with frequent pipettings, made into a homogeneous suspension, and transferred to fresh vessels for subculturing. Therefore, no trypsin digestion or treatment with ethylenediaminetetraacetate (EDTA) was employed.

Although, some of the cells in strain No. 3 appeared to attach to glass surface after establishment of the strain, they were easily removed by frequent pipetting.

RESULTS

Establishment of Cultures

Strain No. 1: In the primary culture, tumor cells grew floating on the concave surface of roller tube and gathering in the center of the surface. They appeared round and there was a large round nucleus with 1 or 2 nucleoli in each cell. Lipid granules were scarecely seen in the homogenous cytoplasm, but increased as the cultivation proceeded.

Among tumor cells were seen very few fibroblast-like cells, which tightly adhered to the wall of the tube and stretched cytoplasmic processes. They seemed to have derived from fibroblasts and histiocytes intermingled with the tumor cells in the ascites. In the first subculture, however, this type of cells was discarded because tumor cells never adhered to glass surface and could be transferred with ease. After 10 days of cultivation, tumor cells in 10 tubes were collected and subcultured.

From the 2nd generation, the culture medium was replaced by the one supplemented with 40% bovine serum. From the 2nd to the 3rd generation, five tubes

were employed for each subculturing and cell multiplication rate decreased gradually. However, marked proliferation of cultivated cells was observed in the 4th generation, on the 58th day. In the 8th generation, cell multiplication rate showed an 18-fold increase of the inoculum in a week, as shown in Fig. 1. Cell number increased

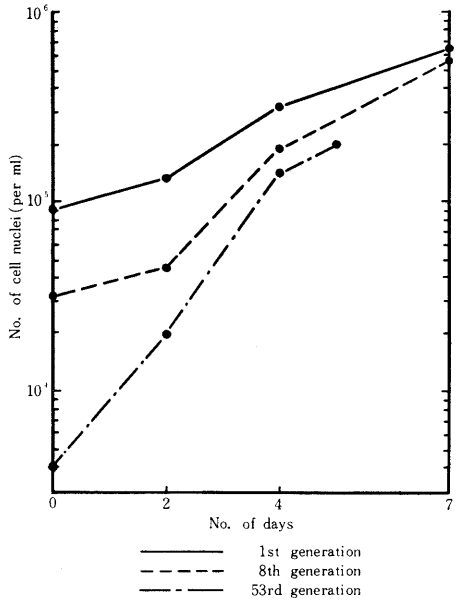

Fig. 1. Growth curves of the strain No. 1 cell in various generations

approximately 50 fold of the inoculum during 5 days in the 53rd generation, on the 322nd day. The multiplication rate has since been constant so that more recent subculture could be made at 5- to 6day intervals. The characteristic features of tumor cells as shown in a phase contrast photomicrograph (Photo 1) have persisted since the establishment of the strain. No. 1 strain has attained a 90th generation, on the 515th day (Table 1)

Table 1. History of Established Strains.

Cell strain	Date of origin (1961)	On Feb.17, 1963		Rate of inoculation (%)
		Generation	Days in total	
No. 1	Sept. 20	90	515	100
No. 2	Sept. 26	89	509	0
No. 3	Oct. 7	87	498	80

Strain No. 2: On the 8th day after initial seeding of ascites into roller tubes, subculture was begun. Characteristic behaviors of cultured cells were similar to those of strain No. 1. Above-mentioned fibroblast-like cells in ascites were removed from the tumor cells on the 1st subculture. At the end of the 2nd generation, on

the 21st day, viable cells were observed in only 2 of 10 tubes and they were carefully transferred to the next generation and cultured for 40 days. In this stage, a definite increase in cell population occurred and thereafter, subculture could be conducted at 5- to 6-day intervals(Photo 2). The tumor cells of this strain have been kept by continuous cultures for more than 89 generation, or 509 days (Table 1).

Strain No. 3: Primary culture of this strain was made for 30 days. During the primary culture, one-half volume of the medium containing the cells in a dilution bottle was discarded and fresh medium was added every 10 days. Sufficient growth occurred towards the end of the primary culture and subculture was then begun. Again, coexistence of few fibroblast-like cells adhering to the bottle wall was observed at the time of seeding of ascites, but they were eliminated from tumor cells on the 1st subculture taking advantage of its adhering property. After weeks of subculture, cell multiplication rate increased rapidly and in the 7th generation, cell nuclei increased 17 fold in a week (Fig. 2).

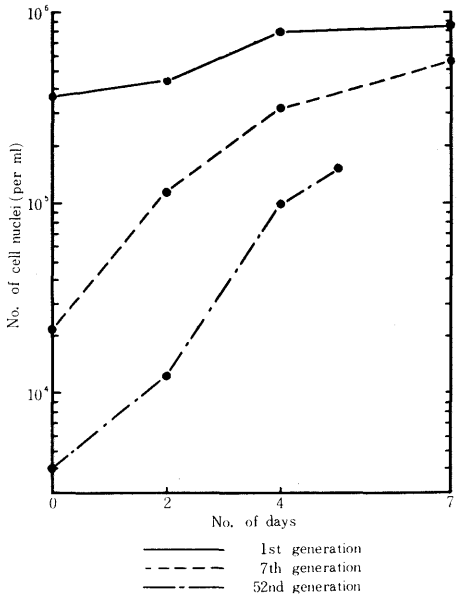

Fig. 2. Growth curves of the strain No. 3 cells in various generations

Microscopically, most of the cells of this strain were larger than those of the other two strains and contained more nucleoli, but there was no significant difference in other features (Photo 3).

After the 26th generation, or 176 days, though there was no obvious change in the medium or external environment, certain amount of the cells came to attach to glass surface within 12 hours after each subculturing and formed small colonies of round cells. However, they spontaneously detached themselves, being alive, from the

glass wall as the growth proceeded and, after frequent pipettings, all cells floating at the bottom were transferred to a subsequent generation with almost the same intervals as used for the other strains. No. 3 strain has attained 87th generation, or 498 days (Table 1).

Inoculation of the Cultured Cell into Mice To determine whether long-term cultivation *in vitro* resulted in a loss of pathogenicity, the 3 strains were inoculated intraperitoneally into NA_2 female mice.

Strain No. 1: After this strain was established, cells from the 13th, 24th, 27th, anb 52nd generations were selected for this study. The cells were harvested after centrifugation at 1,000 r.p.m. for 5 minutes, resuspended in a small amount of saline, counted, and an aliquot containing more than 10^6 cells was injected into mice. A tumor developed in 100% of the animals with typical ascites formation in 5～7 days and the mice died within 2～3 weeks. These cells in ascites closely resembled the parental mouse strain in morphological characterization.

Since the inoculation from the 27th generation, the resulting tumors have been carried intraperitoneally through serial mouse passages. In the first mouse passage, ascites, recognized on the 5th day after inoculation of the cultured cell, was obtained from the inoculated mouse on the 10th day and transplanted to a group of non-treated mice. From the second passage up to the present, the intraperitoneal inoculation has been carried out serially every week. The life span of the inocualted mice was 41 days on an average in the 1st passage, shortened to 25 days in the 2nd, and was later reduced to 14 days which is equal to that in the original mouse-to-mouse passage. These ascites tumors have preserved their histological characteristics, such as cell morphology (Photo 4), enlargement of the liver, spleen, and intraabdominal lymph nodes, massive tumor deposits in the mesentery, and lung metastases.

Strain No. 2: Inoculation was started in the same way as for strain No. 1 and cells were inoculated into mice in the 12th and 25th generations. Tumor incidence was nil with the former material and the mice inoculated with the latter died the next day due to an unknown cause. At this point, transplantability of strain No. 2 cells was regarded lost.

Strain No 3: Inoculation was carried out with the cells from the 13th and 30th generations, or after 107 and 199 days. The inoculated cells elicited ascites tumor within 5 days in all mice used in the former generation, but one-half of the mice survived. On the 7th day after the first inoculation, the ascites tumor derived from an inoculate mouse was transferred intraperitoneally to new NA_2 mice with the cell number of 10^6. All inoculated mice died within 2 weeks. In the test with the 30th generation, the mortality of mice was 100% and the mean survival time was 16 days.

DISCUSSION

The present study has demonstrated the establishment, as continuous cultures *in vitro*, of MN/T and the production of ascites tumor in mice following inoculation of the newly established cell strains.

In many other tumor cell strains established so far, other workers reported various procedures for the propagation of cells because of difficult adaptability to *in vitro* system. For instance, Lettre and Schleich[19] and Sakurai *et al.*[28] obtained a satisfactory culture of Yoshida sarcoma in the presence of fibroblasts, de Bruyn[4] demonstrated a similar relationship in connection with MB strains, Powell[26,27] attempted to promote *in vitro* growth of Ehrlich and Sarcoma 37 ascites tumor cells in coexistence with explants of various animal tissues or normal spleen monocytes, and Wolff[32] obtained luxuriant propagation in the culture of S-180 sarcoma with fragments of normal organs. For the cultivation of Ehrlich ascites carcinoma, Deschner and Allen,[5] and Ely and Gray[7] employed CO_2 gas. Prior to the culture, Guerin and Morgan[12] subjected the cells of Ehrlich ascites carcinoma and 6C3HED mouse lymphosarcoma to a freezing procedure. In the study reported here, however, MN/T cells obtained from the peritoneal cavity of a mouse grew easily on the glass in a fluid medium without any supports such as symbiosis, carbon dioxide, or freezing treatments. The reason for easy adaptation of MN/T to *in vitro* conditions seemed to lie not only in the cell property itself, but in the fact that the medium selected in the previous study[21] was most suitable for the propagation of MN/T.

In the successful establishment of strains, emergence of cell strains from primary cultures was usually followed by a temporary decrease of cell multiplication rate and sudden outburst of growth later.[6,8,9,12,13] Hsu *et al.*,[15~17] Levan and Biesele,[20] and Paul[25] referred to this phenomenon as "transformation" which might involve the selection of competing genotypes particularly suited to the altered environment. These selected cells might exist from the time of isolation or arise in some case as a result of mutations. The same phenomenon termed as "dramatic increase"[8] was also observed in about a month in the course of cultivation of the 3 strains in our study. Thus, the 3 strains have been established and maintained for over 515, 509, and 498 days, respectively, up to the present.

Cells of the three established strains of MN/T have usually grown without adhering to glass surface but floating at the bottom during cultivation. For these advantageous behaviors of the cultured cell, subculturing has been conducted without treatment with trypsin or EDTA. As far as we are aware, few cell strains which demonstrated such a peculiar behavior on growing in a static culture have been reported[16,18,29] besides the present strains.

Recently, cells of strains No. 1 and No. 2 have propagated exponentially as shown by the increase of about 50 fold of the inoculum during 5 days of cultivation. These multiplication rates measured gave a mean division time of approximately 21 hours,

according to the formula introduced by Hauschka *et al.*[13] However, strain No. 3 showed slower multiplication rate (about 40 fold of the inoculum). This difference of growth rate might have been caused by the diverse chromosome constitution among cell strains, as exhibited by Hauschka *et al.*[13, 14] in Ehrlich ascites tumor. In relation to these observations, it was of interest to note that most of the cells of strain No. 3 were larger in size than those of the other strains and contained more nucleoli. Furthermore, preliminary chromosome analysis revealed that the strains No. 1 and No. 2 are near-diploid and the strain No. 3, hypotetraploid.[22] As the culture vessels of the 3 strains, which are of the same origin, have been kept separate from each other since the beginning of culture, each strain would take its own course of evolution, as stated by Hsu and Klatt.[16] Consequently, it was highly possible that various courses of evolution induced the biological differences among these three strains.

In the inoculation studies, cells of strain No. 2 did not induce ascites tumor formation with the inoculum of 10^6 cells which killed the mice in the study with other strains. Many investigators[3, 5, 10, 16, 23, 24,] reported that established malignant cell strains in culture after prolonged residence *in vitro* lost their ability to produce neoplasms when transferred back to the mammals of the appropriate genetic constitution. According to Dawe *et al.*[2] and Hsu *et al.*,[16, 17] one assumption was that the loss of virulence of a cell strain would be elicited with increase in resistance or immune response of the host animal; and secondly, it might be considered, in respect to cell itself, that in making the adaptation to growth *in vitro*, the neoplastic cells acquired metabolic changes that would prove a hindrance when the cells were returned to their former *in vivo* environment. On the other hand, strain No. 1 and No. 3 demonstrated the ability of cells to produce ascites tumors in mice even after prolonged *in vitro* cultivation. Moreover, an interesting finding was obtained in the inoculation with the strain No. 1 cells from the 27th generation. This datum would imply that chromosomal reshuffling occurred within the cell population after inoculation of the cultured cell. Subsequently, cells less favorable to growth *in vivo* might have been eliminated and a subline might have been selected, which adjusted to modification of the environment due to possible metabolic superiority and presented higher malignancy. Thereafter, the subline could have grown rapidly and taken the main part in proliferation to overwhelm the increase of resistance of the host. As for this point of view, their chromosomal analyses were conducted and the result will be discussed in a subsequent paper.[22]

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(Addendum) In the summer of 1962, registration of strain No. 1 was entered at the Japaness Tissue Culture Association and it has since been designated as "JTC-13" with the recognition by the association.

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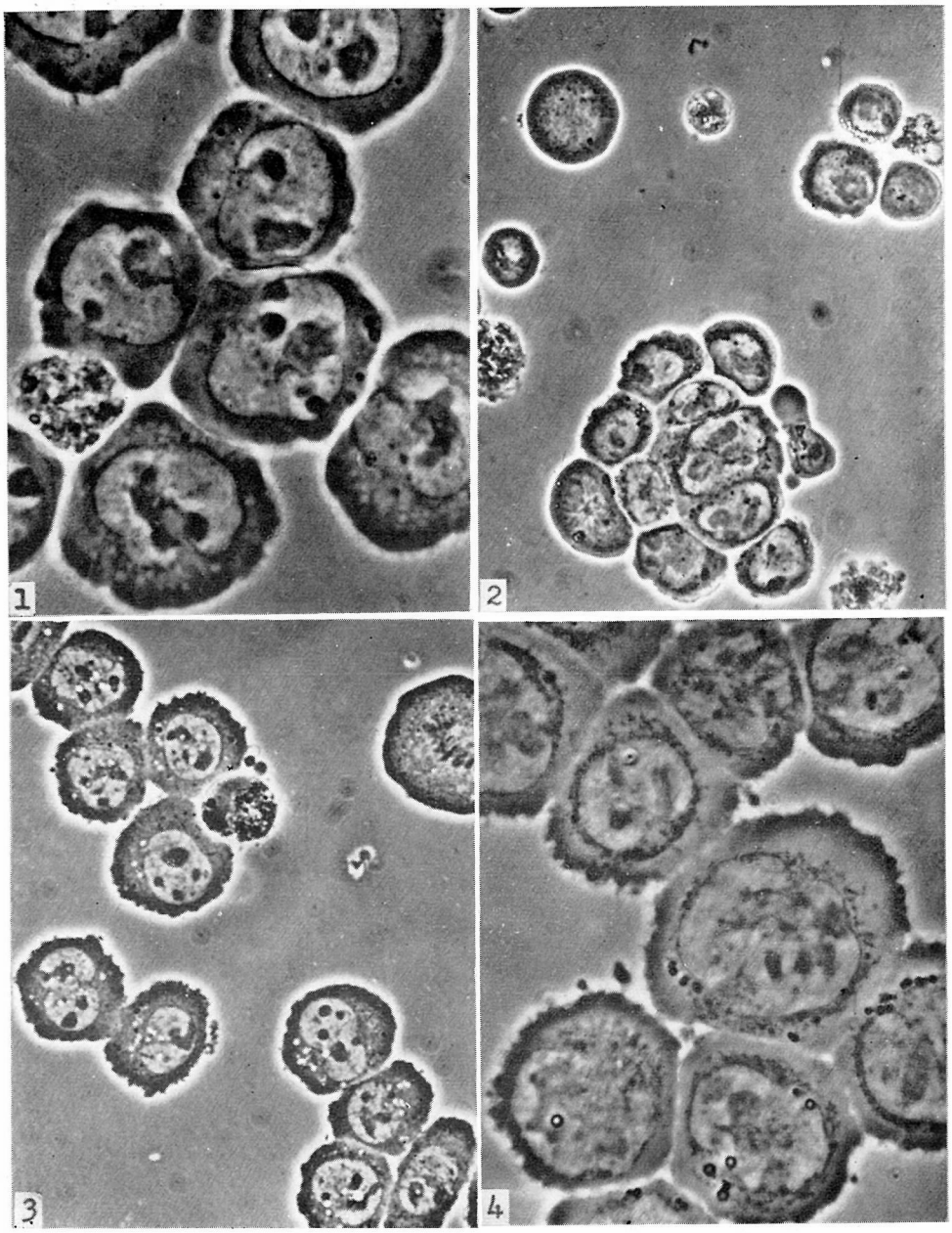
EXPLANATION OF PLATE

Photo 1. Strain No. 1 cells. The volume of the cell is occupied by a large nucleus. Nucleus is approximately round in shape and contains 1~2 nucleoli. Many cytoplasmic granules are scattered around the nucleic membrane. Cytoplasmic membrane is smooth and distinct. Phase. ×1,275.

Photo 2. Strain No. 2 cells. Cytological features are similar to those of strain No. 1. Cells in mitosis are seen. Phase. ×510

Photo 3. Strain No. 3 cells. Note the large size of cells and the increased number of nucleoli, compared with strain No. 2 cells. Phase. ×510.

Photo 4. Ascites tumor cells being maintained by serial mouse-to-mouse passage after the inoculation of strain No. 1 from the 27th generation. Note the similarity of cell morphology between Photos 1 and 4. Phase. ×1,275.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