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Abstract

Three cell lines, spontaneously transformed in vitro, MWE-l and MWE-2 originating from C3Hf/Bi mouse whole embryos and NHLu-l originating from newborn syrian hamster lungs were obtained. These transformed cell lines were characterized by short generation time, marked aneuploidy in chromosome numbers and tumor induction in vivo. Histological appearance of the tumors induced by MWE-l was fibrosarcomatous. Myogenic character in MWE-2 and epithelial character in NHLu-l were interesting features.

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SPONTANEOUS NEOPLASTIC TRANSFORMATION *IN VITRO* OF MOUSE WHOLE EMBRYO CELLS AND NEWBORN HAMSTER LUNG CELLS

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It is necessary for the quantitative analysis of viral carcinogenesis to establish the transformation system of cell lines. For the purpose of experimental research for the neoplastic transformation by adenovirus type 12, the author obtained three established cell lines, two mouse whole embryo cell lines (MWE-1 and MWE-2) and one newborn hamster lung cell line (NHLu-1). These cell lines were, however, spontaneously transformed during prolonged cultivation without virus infection.

Since the works of GEY (1) and EARLE (2), the spontaneous neoplastic transformations *in vitro* have been reported in several species and strains (3, 4, 5, 6, 7). The spontaneous neoplastic transformations usually take place after prolonged cultivation and exact cause and mechanism responsible for transformation have not yet been clarified. In the present work, several characteristics of these cell lines in culture and histology of the tumors obtained by implantation are reported.

MATERIALS AND METHODS

Animal: C3Hf/Bi strain of mice bred in the mouse colony of Okayama University Medical School and Syrian golden hamsters originally obtained commercially and bred in our laboratory were used.

Cell culture: Approximately 17-day-mouse embryos from the same pregnant female were used for the preparation of MWE-1 and MWE-2 cells and newborn hamster lungs were used for the preparation of NHLu-1 cells, respectively.

Lung tissues and embryos were finely minced with scissors, washed in phosphate buffered saline free of Ca^{++} and Mg^{++} (PBS^-), and dispersed by digestion with 0.25 per cent trypsin (Difco 1 : 250) in PBS^- on a magnetic stirrer at 36°C. Trypsin treatment for 40 minutes was repeated twice renewing the trypsin solution.

The dispersed cells were pooled in a centrifuge bottle kept at 4°C, centri-

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fuged at 1,000 r. p. m. for 5 minutes, washed with Earle's solution containing 0.1 per cent yeast extract and 0.5 per cent lactalbumin hydrolysate, seeded into culture vessels (MA-12, Miharu Co., Tokyo) with rubber stoppers, and incubated stationarily at 37°C. Subculture was performed by trypsinization (200 H. U. M. trypsin/ml. Mochida Pharm. Co., Tokyo).

Culture medium: Modified EAGLE's minimum essential medium (MEM) (autoclavable, Nissui Pharm. Co., Tokyo) supplemented with 20 per cent bovine serum was used for the primary and serial cultivations except that MWE-2 cells had been cultivated with Hanks' solution containing 0.1 per cent yeast extract and 0.5 per cent lactalbumin hydrolysate supplemented with 20 per cent bovine serum for initial one month.

Growth curve: When a cell line was established, a desired number of cells was seeded into test tubes (15×150 mm). The medium was removed from three tubes about every 24 hours, the cell layer was scraped off by rubber cleaner after dipping in 0.1 per cent gentian violet solution in 0.1 mol. citric acid more than one hour and pipetted, and the nuclei were counted. The mean number of cells per tube was calculated.

Autoradiography: Generation time (Tc), pre-synthetic gap (T_{G1}), DNA-synthetic phase (Ts), post-synthetic gap (T_{G2}) and mitotic phase (T_M) were determined by pulse-labeling of cells at the logarithmic phase with 0.1 μC ^{3}H-thymidine/ml. for 30 minutes and by analysing the changes with time in per cent labeled mitosis (8). The coverslips were fixed with absolute methanol, dipped in liquid emulsion (Sakura Co., NR-M1), at 45°C, stored in the dark box at 4°C for an appropriate length of time, and developed with Konidol X (Sakura Co.).

After being stained with Giemsa and mounted with bioleit, the slides were scored for labeled cells.

Chromosome studies: Colchicine solution (0.5 mg per cent) was added to the cell culture at the logarithmic phase for 6 to 12 hours. The cells were dispersed with trypsin and then centrifuged. The trypsin was removed and the hypotonic solution of NaCl (0.17 per cent) had been added for 30 minutes. After the hypotonic solution of NaCl was removed, the cells had been fixed twice with Carnoy's solution renewing the fixatives for 10 minutes and 20 minutes, respectively. The cells were centrifuged again and placed on the cold slides. The cells were dried by hot air and stained with Giemsa.

Transplantation: Cell cultures were trypsinized, sedimented, counted, and then resuspended in MEM at a desired density. MWE-1 and MWE-2 cells were implanted subcutaneously into 20- to 30-day-old male C3Hf/Bi mice, and NHLu-1 cells were implanted subcutaneously into 2-month-old male hamsters.

Histological examination: Tumor tissues were fixed in 20 per cent neutral formalin solution and stained with hematoxylin and eosin. If necessary, Mallory-Azan stain, Mallory's phosphotangustic acid hematoxylin stain (PTAH), PAS reaction and Pap's silver impregnation were performed.

RESULTS

History of the cell lines:

MWE-1: This cell line originated from approximately 17-day-mouse whole embryos of both sexes. The cells had propagated slowly until about 230 days from the start (the 10th passage) and then the cell growth stopped completely. About 360 days after the start, the cell proliferation became well again and short spindle-shaped or polygonal cells grew predominantly (Photo 1).

MWE-2: This cell line originated from the same materials to those used to MWE-1. The growth rate was low and the populations mainly consisted of fibroblastic cells and macrophages in the early period of cultivation. Fibroblastic cells have become predominant and grown well since about 200 days from the start (the 5th passage) (Photo 2).

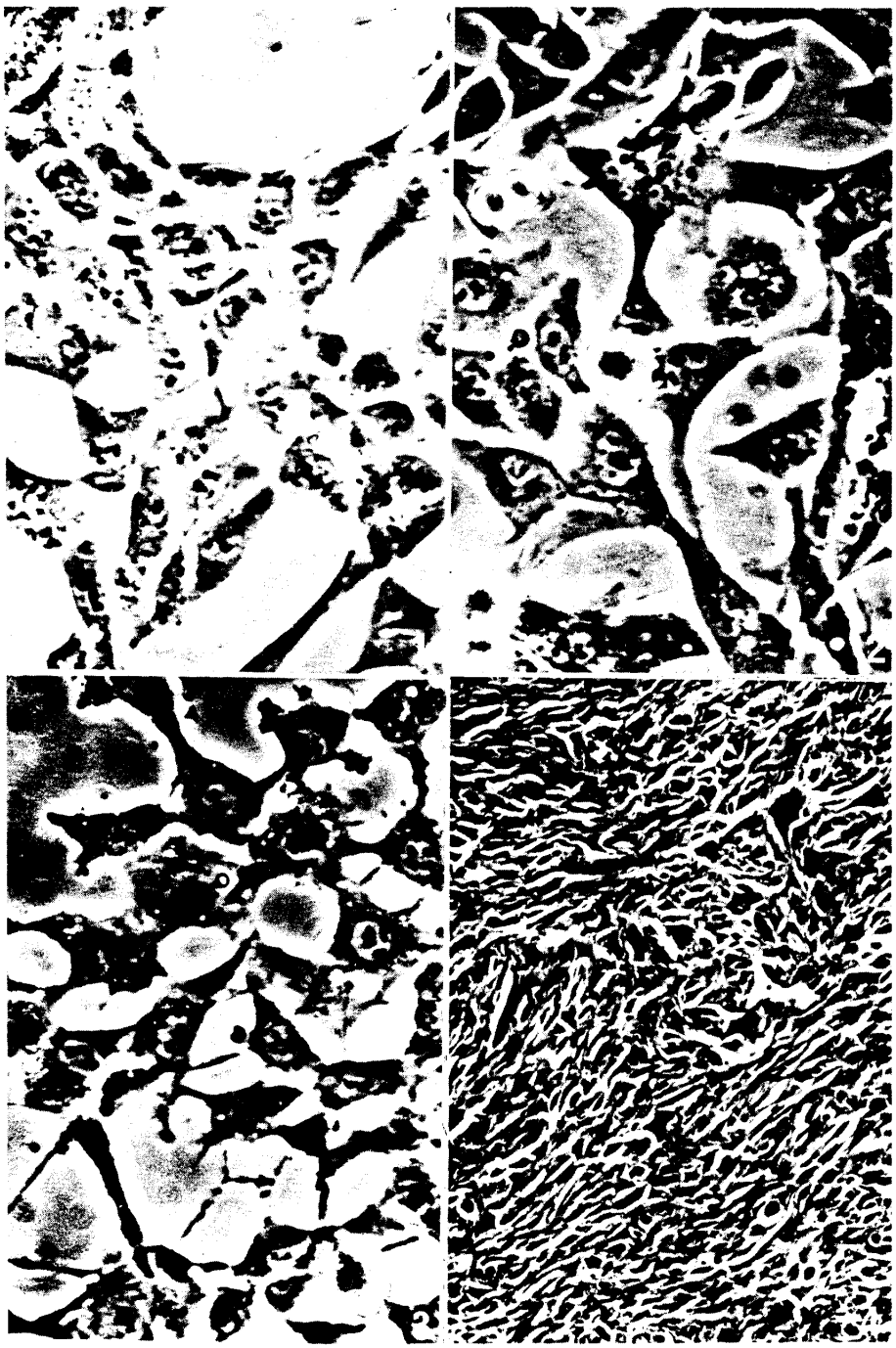
NHLu-1: This cell line originated from the lung tissues of newborn hamsters of both sexes. The growth rate was low and soon after the start the cell growth stopped. The culture mainly consisted of fibroblastic cells and epithelial cells in the early period of cultivation. The rate of cell proliferation became high and polygonal cells with fine digitate cytoplasmic processes became predominant about 330 days after the start (the 4th passage) (Photo 3). Each cell line has been subcultured every 4 to 7 days since establishment as a stable cell line.

Chromosome analysis: The distribution of chromosome numbers in the different cell lines are represented in Fig. 1. All cell lines showed marked aneuploidy. The modal number of chromosomes was 68 (at the 74th passage) and 70 (at the 92nd passage) in MWE-1 cells, 101 (at the 73rd passage) and 98 (at the 95th passage) in MWE-2 cells, and 40 (at the 28th passage) and 44 (at the 84th passage) in NHLu-1 cells. Karyotypic analysis was not performed.

Growth rate: The doubling time ranged from 12 to 18 hours. MWE-1 had a doubling time of 12 hours, MWE-2 of 18 hours and NHLu-1 of 12 hours (Fig. 2).

Analysis of cell cycle: Cell cycle was determined by pulse labeling-chasing method (Fig. 3). MWE-1 had a generation time (T_C) of 13.4 hours, T_{G1} of 1.9 hours, T_S of 8.7 hours, T_{G2} of 2.4 hours and T_M of 0.4 hours. MWE-2 had T_C of 13.5 hours, T_{G1} of 1.8 hours, T_S of 8.2 hours, T_{G2} of 2.8 hours and T_M of 0.7 hours. NHLu-1 had T_C of 11.8 hours, T_{G1} of 3.2 hours, T_S of 5.8 hours, T_{G2} of 2.2 hours and T_M of 0.6 hours.

Transplantation test: All cell lines produced tumors by subcutaneous



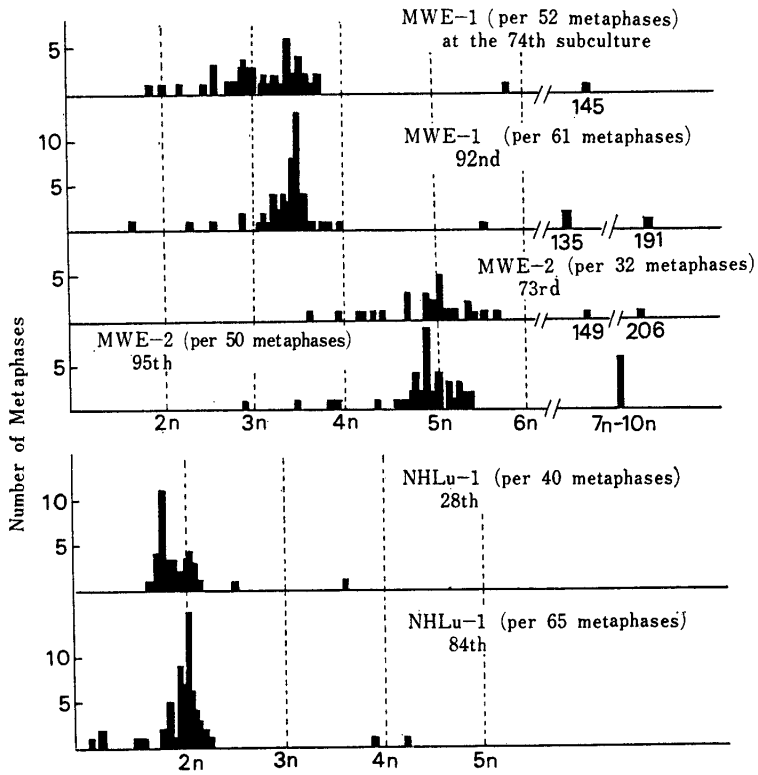


Fig 1. Distribution of chromosome numbers

inoculation into the animals. The results of the transplantation are shown in Table 1.

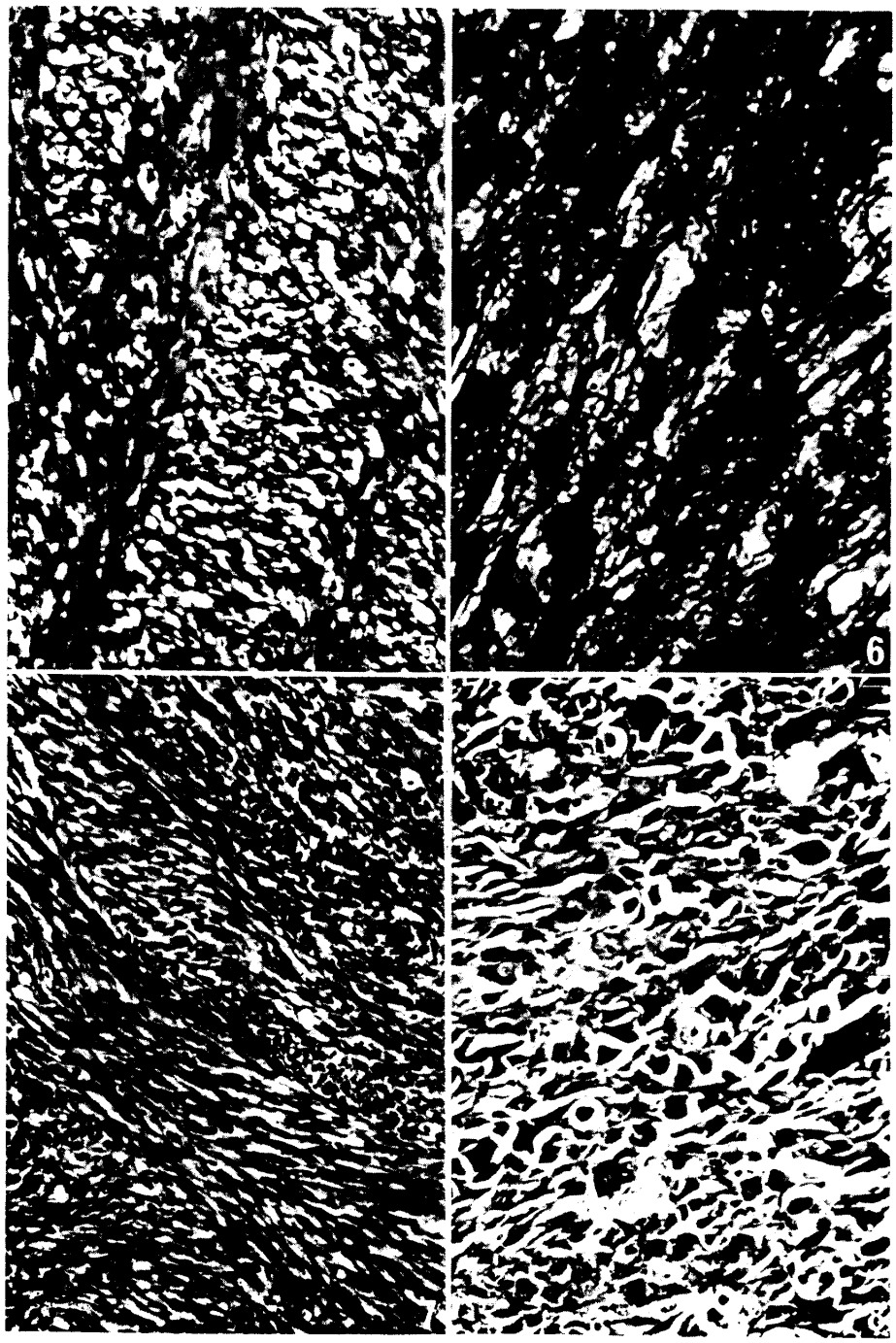
Histomorphology of the tumors obtained by implantation of cells: Tumors induced by MWE-1 cells showed fibrosarcomatous characteristics composed of irregular or interlacing arrangements of spindle-shaped cells with

Photo 1. Short spindle-shaped or polygonal cells with fine cytoplasmic processes make the monolayer sheet, but some extent of overlapping of cytoplasmic processes is seen.
MWE-1. Phase contrast. × 360

Photo 2. Fibroblastic cells with digitate cytoplasmic processes form loose networks.
MWE-2. Phase contrast. × 360

Photo 3. Polygonal cells with clear nucleoli and abundant cytoplasmic granules show loose monolayered network. Fine digitate cytoplasmic processes are clearly seen.
NHLu-1. Phase contrast. × 360

Photo 4. Fibrosarcomatous area showing marked, irregular proliferation of the pleomorphic spindle-shaped tumor cells.
MWE-1 tumor. H-E. ×180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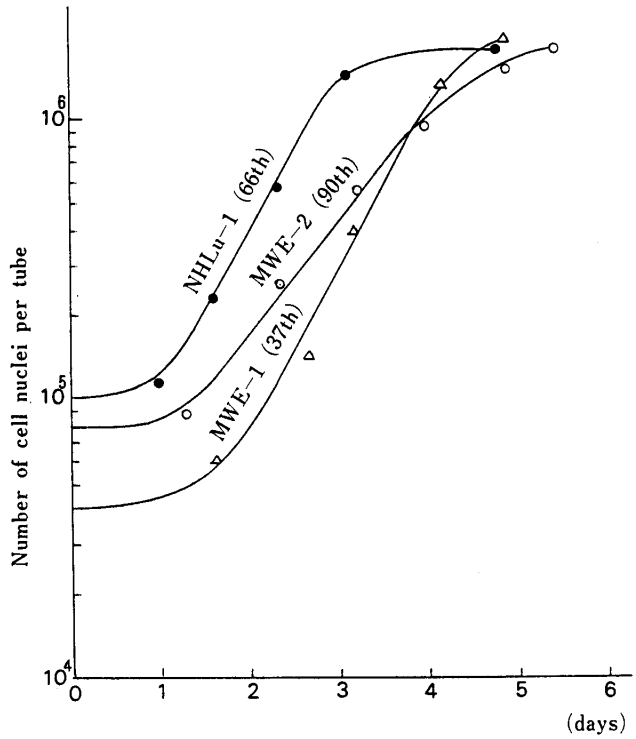

Fig 2. Growth curves

marked pleomorphism (Photos 4 and 5). Silver impregnation showed abundant irregular production of reticulin fibers (Photo 6). Tumors induced by MWE-2 cells consisted of interlacing and bundle forming proliferation of spindle-shaped cells, intermingled with giant cells in some areas (Photos 7 and 8). Silver impregnation showed abundant reticulin fiber formation which encircled individual cells incompletely (Photo 9). These findings were somewhat different from usual fibrosarcoma, suggesting

Photo 5. Fibrosarcomatous area with interlacing bundle formation of the spindle-shaped tumor cells.
MWE-1 tumor. H-E. × 180

Photo 6. Abundant irregular reticulin fibers are seen associated with or among the tumor cells.
MWE-1 tumor. Pap's silver impregnation. × 720

Photo 7. Irregular fascicular and interlacing proliferation of the spindle-shaped tumor cells.
MWE-2 tumor. H-E. × 180

Photo 8. Tumor cells show a marked pleomorphism with many multinuclear giant cells.
MWE-2 tumor. H-E. × 180

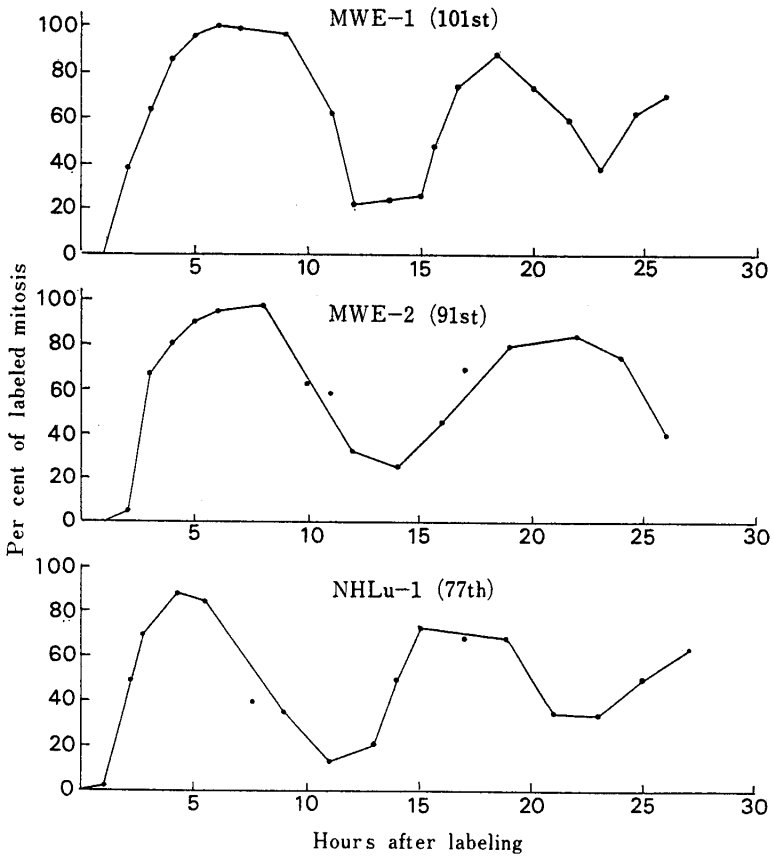

Fig 3. The cell cycle

the presence of myogenic component, although there was no definite evidence.

NHLu-1 cells produced peculiar tumors composed of large polygonal cells embedded among the stromal cells and forming cell sheets of various

Photo 9. Reticulin fibers encircle one or two tumor cells. MWE-2 tumor. Fap's silver impregnation. × 720

Photo 10. One or two polygonal tumor cells are embedded among the abundant connective tissue.
NHLu-1 tumor. H-E. × 180

Photo 11. Tumor cells are polygonal and form the cell nests. Reticulin fibers can hardly be detected among the tumor cells.
NHLu-1 tumor. Pap's silver impregnation. × 720

Photo 12. Tumor cell nests are encircled by the connective tissue and reticulin fibers production among the tumor cells is very scanty.
NHLu-1 tumor. Pap's silver impregnation. × 180

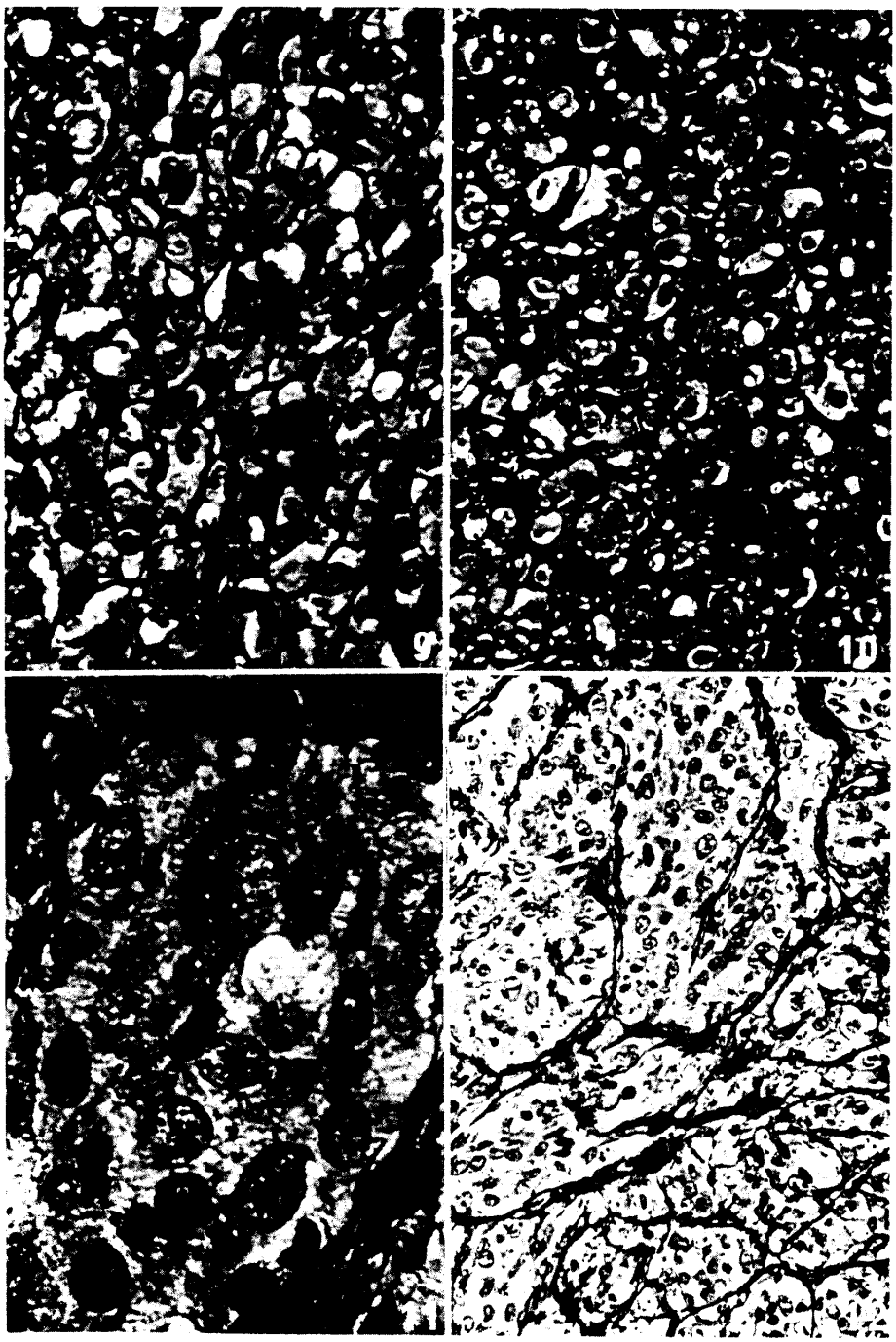
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TABLE 1. TUMORIGENICITY

Cell line	Passage No.	Inoculum Size	No. of animals that produced tumors* / No. of animals inoculated
MWE-1	32	1×10^5	3/3
	(482)**	1×10^6	1/1
MWE-2	23	5×10^4	2/3
	(392)	5×10^5	2/3
	36	5×10^4	3/3
	(482)	5×10^5	2/3
NHLu-1	23	2×10^5	2/2
	(488)	2×10^6	2/2

* Decision of the tumor production was done 3 months after the transplantation.
** Numbers within parentheses show culture days.

size. In some parts, only one or two cells were surrounded by the stromal cells (Photo 10).

Tumor cells had clear nucleoli. Reticulin and collagen fibers could hardly be detected among the tumor cells and they encircled the tumor cell nests (Photos 11 and 12). These findings suggest a kind of unclassified malignant epithelial tumor, but a definite diagnosis could not be made.

DISCUSSION

C3Hf/Bi mouse whole embryo cells were spontaneously transformed and produced malignant tumors by implantation into the syngeneic host after long term cultivation. Mouse cells have been found to be relatively easily transformed *in vitro* usually after prolonged cultivation (6, 7, 9, 10, 11, 12).

Transformation is not only restricted to the mouse cells, but transformation took place also in the hamsters, especially in the lung tissues (13, 14). In the present experiment, newborn hamster lung tissues were spontaneously tranformed, too. Prolonged time is usually required for neoplastic conversion (5, 15). All cell lines reported here also required much time, 482 days to MWE-1, 392 days to MWE-2 and 488 days to NHLu-1.

However, it is known that in sparse cultures of hamster cells, morphologically transformed colonies appeared already in primary cultures (14). The experiment using clonal assay of the primary culture is required for the more precise and critical analysis of the transformation process. Both of the transformed mouse cell lines, MWE-1 and MWE-2 showed

heteroploidy of chromosomes consistent with other investigator's observation (7, 16). Some investigators observed the appearance of chromosome abnormalities preceding the neoplastic conversion (7, 17), but we have no available data to the chromosome numbers of the preneoplastic stage. NHLu-1 had chromosome numbers of diploid or near-diploid range. Diploid or neardiploid neoplastic cell lines with abnormal chromosome have been observed (12, 13), but karyotypic analysis of NHLu-1 was not performed. Generation time of these cells was rather short compared with other cell lines in the literature (18).

The tumors induced by implantation of spontaneously transformed cells have been usually fibrosarcoma (7, 10, 19) with a few exceptions (20, 21). In the present studies, only one strain, MWE-1 induced fibrosarcoma and the other two strains, MWE-2 and NHLu-1 induced the tumors with the peculiar histological patterns different from usual fibrosarcoma, suggesting the presence of myogenic and epithelial components, respectively. Morphology of the cells *in vitro* and *in vivo* are compatible in some extent. However, the possibility that several tissue components are intermingled or one cell type makes tumor selectively cannot be denied, because cloning of these cells have not yet been performed.

SUMMARY

Three cell lines, spontaneously transformed *in vitro*, MWE-1 and MWE-2 originating from C3Hf/Bi mouse whole embryos and NHLu-1 originating from newborn syrian hamster lungs were obtained. These transformed cell lines were characterized by short generation time, marked aneuploidy in chromosome numbers and tumor induction *in vivo*. Histological appearance of the tumors induced by MWE-1 was fibrosarcomatous. Myogenic character in MWE-2 and epithelial character in NHLu-1 were interesting features.

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REFERENCES

1. GEY, G. O.: Cytological and cultural observations on transplantable rat sarcomata produced by the inoculation of altered normal cells maintained in continuous culture. *Cancer Res.* **1**, 737, 1941
2. EARLE, W. R. and NETTLESHIP, A.: Production of malignancy *in vitro*. V. Results of injections of cultures into mice. *J. Nat. Cancer Inst.* **4**, 213, 1943

3. STOKER, M. and MACPHERSON, I.: Syrian hamster fibroblast cell line BHK21 and its derivatives. *Nature* **203**, 1355, 1964
4. GOTLIEB-STEMATSKY, T., YANIV, A. and GAZITH, A.: Spontaneous malignant transformation of hamster embryo cells *in vitro*. *J. Nat. Cancer Inst.* **36**, 477, 1966
5. DIAMOND, L.: Two spontaneously transformed cell lines derived from the same hamster embryo culture. *Int. J. Cancer* **2**, 143, 1967
6. SANFORD, K. K., EARLE, W. R., SHELTON, E., SCHILLING, E. L., DUCHESNE, E. M., LIKELY, G. D. and BECKER, M. M.: Production of malignancy *in vitro*. XII. Further transformations of mouse fibroblasts to sarcomatous cells. *J. Nat. Cancer Inst.* **11**, 351, 1950
7. BARSKI, G. and CASSINGENA, R.: Malignant transformation *in vitro* of cells from C57BL mouse normal pulmonary tissue. *J. Nat. Cancer Inst.* **30**, 865, 1963
8. YAMADA, M.: *Tanpakushitsu Kakusan Kôso* **12**, 838, 1967 (in Japanese).
9. EVANS, V.J., PARKER, G.A. and DUNN, T.B.: Neoplastic transformation in C3H mouse embryonic tissue *in vitro* determined by intraocular growth. I. Cells from chemically defined medium with and without serum supplement. *J. Nat. Cancer Inst.* **32**, 89, 1964
10. ANDRESEN, W. F., EVANS, V. J., PRICE, F, M. and DUNN, T. B.: Neoplastic transformations in cells explanted from the kidneys of 3-day-old C3H mice. *J. Nat. Cancer Inst.* **36**, 953, 1966
11. EVANS, V. J. and ANDRESEN, W. F.: The effect of serum on spontaneous neoplastic transformation *in vitro*. *J. Nat. Cancer Inst.* **37**, 247, 1966
12. SANFORD, K. K., BARKER, B. E., PARSHAD, R., WESTFALL, B. B., WOODS, M. W., JACKSON, J. L. and KING, D. R.: Neoplastic conversion *in vitro* of mouse cells: cytologic, chromosomal, enzymatic, glycolytic, and growth properties. *J. Nat. Cancer Inst.* **45**, 1071, 1970
13. OKUMURA, H.: Spontaneous malignant transformation of hamster lung cells in tissue culture. In H. KATSUTA (ed), Cancer Cells in Culture, p. 292, University of Tokyo Press, Tokyo, University Park Press, Baltimore and State College, Pennsylvania, 1968
14. YANIV, A. and GOTLIEB-STEMATSKY, T.: Factors associated with spontaneous transformation of hamster cells in culture. *J. Nat. Cancer Inst.* **44**, 283, 1970
15. SANFORD, K. K. and HAEMANN, R. E.: Neoplastic transformation of mouse and hamster cells *in vitro* with and without polyoma virus. *J. Nat. Cancer Inst.* **39**, 691, 1967
16. PARSHAD, R. and SANFORD, K. K.: Effect of horse serum, fetal calf serum, calf serum, bovine serum, and fetuin on neoplastic conversion and chromosomes of mouse embryo cells *in vitro*. *J. Nat. Cancer Inst.* **41**, 767, 1968
17. EVANS, V. J., JACKSON, J. L., ANDRESEN, W. F. and MITCHELL, J. T.: Chromosomal characteristics and neoplastic transformation of C3H mouse embryo cells *in vitro* in horse and fetal calf serum. *J. Nat. Cancer Inst.* **38**, 761, 1967
18. YAMADA, M.: *Tanpakushitsu Kakusan Kôso* **9**, 1153, 1964. (in Japanese)
19. CORNELL, R.: Spontaneous neoplastic transformation *in vitro*: Ultrastructure of transformed cell strains and tumors produced by injection of cell strains. *J. Nat. Cancer Inst.* **43**, 981, 1969
20. EVANS, V.J., HAWKINS, N.M., WESTFALL, B.B. and EARLE, W.R.: Studies on culture lines derived from mouse liver parenchymatous cells grown in long-term tissue culture. *Cancer Res.* **18**, 261, 1958
21. SATO, J., NAMBA, M., USUI, K. and NAGANO, D.: Carcinogenesis in tissue culture. VIII: Spontaneous malignant transformation of rat liver cells in long-term culture. *Japan. J. Exp. Med.* **38**, 105, 1968