Chromosomes and Some Properties of a Friend Virus-induced Ascites Tumor Grown in Culture[1)]

By

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(With 12 Text-figures and 1 Table)

To date, evidence has considerably been gathered that transplantable Friend virus-associated tumor variants of mcie could be introduced in culture and that cells retained their properties during a long period of serial *in vitro* passages (Fieldsteel *et al.* 1966). Quite recently, Sofuni *et al.* (1967) reported that two lines of transplantable Friend virus-induced tumors of mice in ascites form (Oboshi *et al.* 1964) were characterized by stable stemline karyotypes both morphologically and numerically.

The present study deals with 1) the *in vitro* cultivation of cells from a line of transplantable Friend virus-induced ascites tumor which was referred to as a "non-virus producing Friend tumor" (NVPFT) (Kobayashi *et al.* 1966), 2) the influence of serial *in vitro* passages on the chromosome pattern of tumor cells under rigid laboratory conditions, and 3) the tumor producing capacity of the *in vitro* cells in susceptible mice under an alteration of stemiline chromosomes.

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Material and Methods: A line of a non-virus producing Friend tumor (NVPFT) here considered was derived from Friend virus-induced tumors of dd/0m mice (Kobayashi *et al.* 1966). The tumor used for culture was an ascites tumor which had been maintained over 120 passages in the ascites form in mice. One ml of viscous ambor-colored ascites fluid was drawn from a tumor mouse. After centrifugation at 100 rpm for 5 minutes, the supernatant was discarded and the cells were resuspended in 3 ml of the medium consisting of 80 per cent 109 medium and 20 per cent of inactivated calf serum. Two TD-15 flasks were seeded with 1.5×10^7 cells and incubated at 37°C. After 48 hours the color of fluids was turned into yellowish orange. Microscopic examination revealed that most cells were suspended in the medium and a relatively few had settled on the glass and flattened out. The cell containing medium was poured into centrifuge tubes and spun at 100 rpm for 5 minutes. The old medium was decanted. The cells were resuspended in 20 ml of the medium and

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transferred to two TD-40 flasks. Since cells were not adhesive, the above procedures made serial passages possible.

For growth curve studies, freshly harvested cells were suspended in 200 ml of the growth medium, and were transferred to TD-40 flasks, 10 ml in amount. They were selected at random for study. Individual cell suspensions were centrifuged at 100 rpm for 5 minutes and resuspended in 5 ml of the medium for cell counts with haemocytometer. The procedures were repeated at 24-hour intervals for 9 days.

Cultured cells taken 24 hours after initiation of cultivation were pretreated with 5×10^{-7}M colchicine for 2 hours and with a hypotonic solution (0.5% sodium citrate) for 15 minutes. Chromosome slides were made according to the routine air-drying method and stained with Giemsa.

For observation of living cells, a 2 ml cell suspension containing 10^6 cells was inoculated into a Rose chamber, and incubated at 37°C for 24 hours. Observations were carried out with phase optics.

At the 10th passage in culture, the cells were collected in a centrifuge tube. After centrifugation, the cells were washed twice with fresh medium and diluted to 10^6/0.2 ml with the fresh medium without serum. Five mice of dd/0m strain received intraperitoneal inoculations with the diluted suspension. After 9 days, the ascites tumor was derived from the inoculated mice and the chromosomes were observed according to the routine air-drying method.

Results

1) *In vitro* characteristics of tumor cells: The cultures have been maintained for 3 months without difficulty over 16 serial passages. The cells and their capacity for culture have remained stable. Figure 1 represents a growth curve of cultured

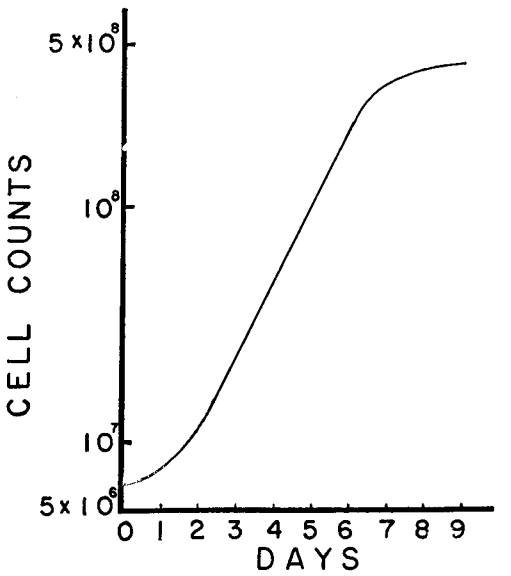


Fig. 1. Growth curve of Friend virus-induced sarcoma in tissue culture.

cells. Plotting cell counts indicate that cell division was logarithmic between the 2nd and 6th day. The increase in cell number was almost 100-fold and the population doubling time estimated from the curve was 22.8 hours.

Most cells were not adhesive to the glass but reproduced, and maintained themselves in suspension without agitation (Fig. 2). The cells were spherical, ranging in size from 10 to 15 micra with a mean of 14 micra. A single round nucleus with 2 to 4 large irregularly shaped nucleoli occurred in most cells. The cytoplasm formed a narrow rim around the nucleus. A large fat droplet was visible in the cytoplasm. Most spherical cells showed blebs or projections around the periphery. With phase optics it was shown that the blebbing continued within a few minutes and reappeared in a different place along the cell surface. Occasionally the blebs were observed pinch off. Generally the cells were growing in suspension, but a small number of them (less than 0.1%) attached to the glass with a flattened and epithelial-like growth. They showed many granules and small vacuoles in the cytoplasm (Figs. 4 and 5). Occassionally, contiguous groups of spherical cells came to join with these flattened cells (Figs. 3 and 4). In Giemsa-staining-preparations, the nuclei showed a fine reddish-purple chromatin network in the spherical cells. The cytoplasm of glass attached-flattened cells stained purple blue paler than that of the spherical ones, suggesting a less basophilic nature (Fig. 6).

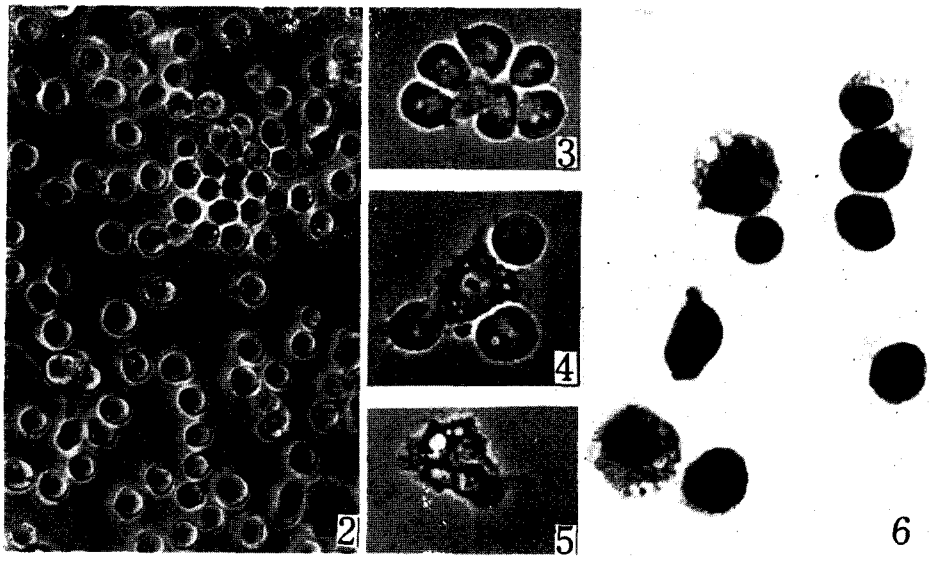
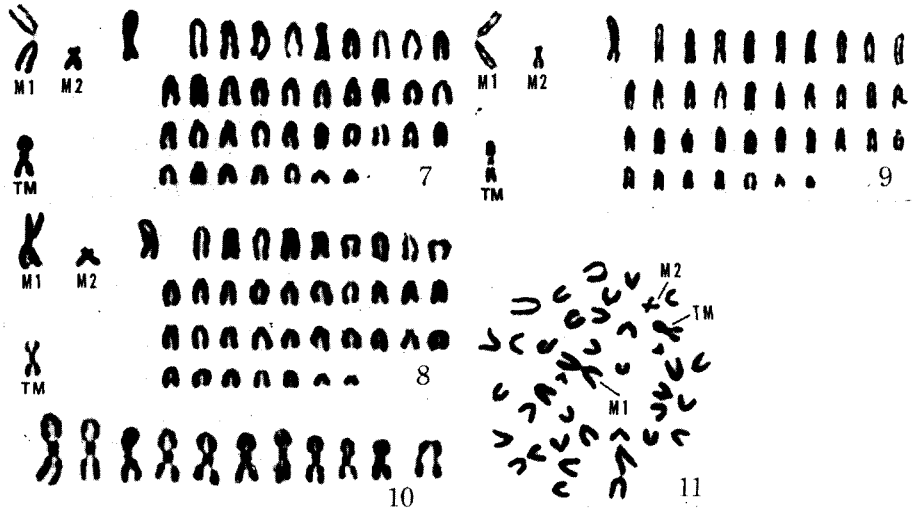


Fig. 2. Phase-contrast picture of ascites tumor cells at the 10th *in vitro* passage. ×200. Figs. 3–5. Phase-contrast pictures of ascites tumor cells attached to the culture vessel. ×400. 3–4: Flattened cells associated with round cells. 5: A flattened cell attached to the glass surface. Fig. 6. Fixed and stained cells, from the 10th *in vitro* passage.

2) Chromosome analyses in cultured cells: The chromosome number of the stemline cells in culture taken 24 hours after the initiation of cultivation was 40 forming a mode (Figs. 7 and 12). The karyotype analysis revealed that an outstanding and unusual telocentric chromosome occurred in the modal cells. This telocentric marker (TM) (Fig. 10) was of remarkably large size, though not larger than the largest element. It showed a secondary constriction on each chromatid which located close to the middle portion of the body. The two chromatids were adherent to each other in this region in the majority of cells observed (in 98% of the 100 TM chromosomes studied). In addition, there were one large metacentric



Figs. 7–9. Karyotypes of cells *in vitro*. 7: 40 chromosomes including 3 prominent markers (M1, M2, and TM); 0 passage, 24 hours after initiation of culture. 8: 40 chromosomes including 3 markers (M1, M2, and TM), from the 7th *in vitro* passage. 9: 40 chromosomes including 3 markers (M1, M2, and TM), from the 10th passage. Fig. 10. A remarkable marker (TM) chromosome, each one from 11 different metaphase cells. Fig. 11. An ascites tumor cell at metaphase, from a reinoculated mouse.

chromosome and one small submetacentric one, being very prominent in the complement. It is then evident that the stemline karyotype of this culture line is characterized by the presence of a TM chromosome, a large metacentric chromosome (M1), and a small submetacentric one (M2), in addition to the remaining 37 telocentric chromosome (Fig. 7). The incidence of cells with the TM chromosome taken 24 hours after cultivation was as shown in Table 1 and Figure 7. The incidence of cells having the TM chromosome showed a gradual increase in the course of serial *in vitro* transfers for 3 months. The large metacentric and the small submetacentric made their constant appearance in every cell so far observed.

Table 1. Chromosome analysis in the Friend virus-induced tumor grown in culture

Passage	Distribution of chromosome number								Incidence of cells with TM
	34-38	39	40	41	42	43	50-57	77-82	
0	2	5	16	9					20/31 (64.5%)
7th	2	8	25	8	4	2	3	3	40/50 (80.0%)
10th	1	6	24	10	5	2	1	2	39/51 (76.5%)
15th	1	6	18	5					27/30 (90.0%)

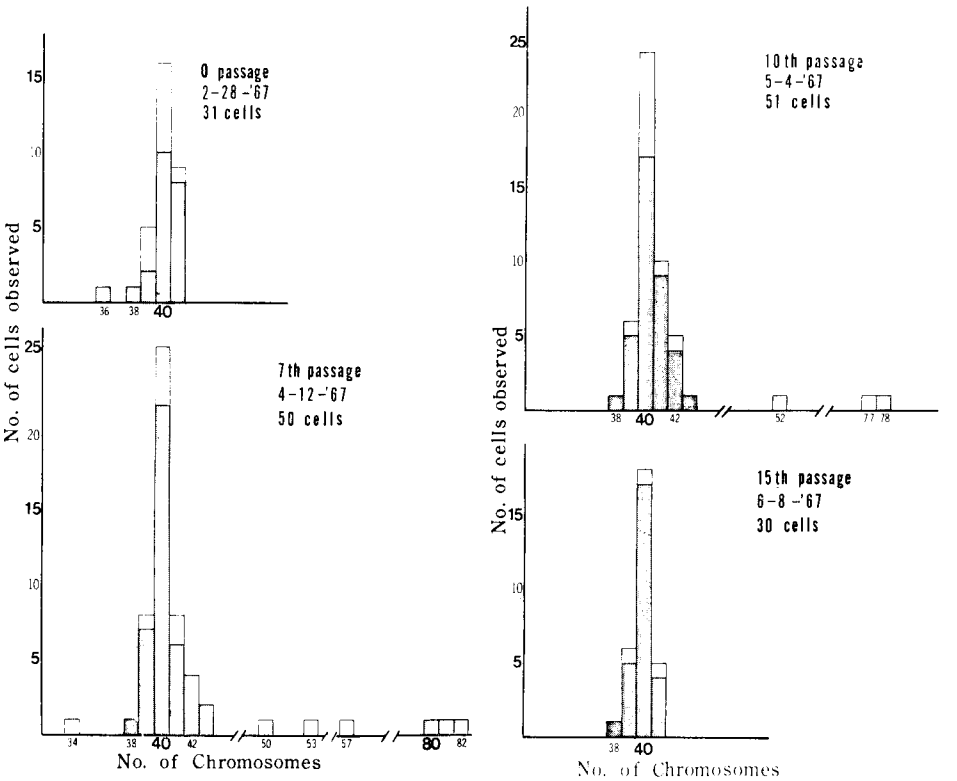


Fig. 12. Distribution of chromosome numbers in cultured cells of Friend virus-induced ascites tumor (NVPFT), from the 0, 7th, 10th and 15th passage. White column indicates cells without the TM chromosome.

Investigations of the *in vitro* cells during serial transfers revealed that there is a general similarity of the stemline chromosomes between the 1st passage cells and later passage cells. Morphologically, the chromosomes showed no alteration between them, though there was a narrow variation in number of chromosomes in both sides of the mode (Figs. 7, 8, 9, and 12). The relative frequency of cells with a TM chromosome was 80.0 per cent in the 7th passage, 76.5 per cent in the 10th passage and 90.0 per cent in the 15th passage (Table 1). There is a tendency showing that the incidence of cells with a TM chromosome increases with the increase of transplant generations.

Cells having 50 to 57 chromosomes occurred in a low frequency in the 7th to 10th passages. They showed no TM chromosome.

The cells from the 12th *in vitro* passage were inoculated into 5 mice of dd/0m strain. It was found that the cells had the capacity to produce a tumor in ascites form in each case. Chromosome observations were made on cells from the ascites tumors produced, and made it clear that the chromosome pattern was similar in cells from the 12th passage and in those from early passages. The stemline karyotype consisted of 3 outstanding markers and 37 telocentrics (Fig. 11). The frequency of the cells having the TM marker was 85.0 per cent.

Discussion

The present study indicated that Friend virus-induced ascites tumor (NVPFT) grew *in vitro* in a manner similar to that reported by Fieldsteel *et al.* (1966) in the Friend virus-induced ascites tumor variants and by Fischer (1958) in the L-5178 strain of lymphocytic mouse leukemia. Most cells in culture were found to be spherical in shape and showed an active cytoplasmic blebbing which appeared to be similar to the cytoplasmic bubbling described by Costero and Pomerat (1951), Rose *et al.* (1963) and Fieldsteel *et al.* (1966). It was found that a small number of cells in culture (less than 0.1%) attached to the glass and appeared as flattened and epithelial-like cells to which some spherical cells tended to aggregate as contiguous groups. Over 10 passages the tumor cells grew in culture with a striking increase of cell numbers.

Sofuni *et al.* (1967) studied the chromosomes of stemline cells in Friend virus-induced mouse ascites tumors, and found that the stem-cells had a pseudo-diploid chromosome constitution, 40 in number and were characterized by 4 marker chromosomes, a large metacentric, a small submetacentric, a large acrocentric and a small acrocentric chromosome. The tumor cells grown in serial cultures showed a remarkable alteration in stelmline chromosomes. The stemline karyotype consisted of 37 telocentrics and 3 prominent markers which were represented by 1 large telocentric, 1 large metacentric and a small submetacentric one. The difference of stemline karyotypes is thus very striking between the tumors of an ascites form and those grown in culture.

In this connection, the findings of Wald *et al.* (1964) in mouse leukemia are

worth mentioning. In bone marrow cells of leukemic mice which had been injected previously either with leukemic spleen cells or with cell-free ultracentrifugates, they noted the consistent occurrence of an extra chromosome and of a marker chromosome with achromatic staining portion on each chromatid situated about two-thirds of the distance from the centromere to the distal end.

The incidence of cells with the TM marker chromosome increased gradually from 64.5 per cent to 90 per cent in the course of serial transfers for about 3 months. This seems to be the result of an adaptation and selection taking place in cells soon after the karyotype alteration. Similar examples have been reported in ascites cell populations of rats and mice (Hsu and Klatt 1959, Okumura *et al.* 1958, Ely and Gray 1961, DiPaolo 1962, De Bruyn and Hansen-Melander 1962).

It was shown by the present study that the cells grown in culture for more than 4 months had the ability to produce reticulum cell sarcomas indistinguishable from the original tumors when inoculated into susceptible mice. Fieldsteel *et al.* (1966) reported similarly that cells grown *in vitro* through more than 2 years still retained their original characteristics such as the ability to produce tumors when inoculated into mice. The stemline chromosomes of ascites tumor cells after the inoculation into mice were morphologically similar to those from cells grown in culture, being remarkable by the occurrence of 3 markers such as a TM chromosome, a large metacentric, and a small submetacentric.

Summary

An ascities tumor variant of the Friend virus-induced tumor (NVPFT) was cultivated in tissue culture. The cells grew in culture with 22.8 hours for one generation. They retained the ability to produce tumor morphologically indistinguishable from the original tumor. The tumor cells grown in culture showed an alteration of the stemline chromosomes from those in ascites form, and were characterized by 3 prominent marker chromosomes: The stemline karyotype showed 40 chromosomes which consisted of a very large telocentric chromosome, a large metacentric, a small submetacentric, in addition to 37 telocentric chromosomes.

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