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Title	Cytological Studies of Tumors, XXXVII. : Chromosome Conditions of Animal Tumors after Deep Freezing (With 16 Text-figures)
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Citation	北海道大學理學部紀要, 14(4), 576-588
Issue Date	1961-12
Doc URL	https://hdl.handle.net/2115/27337
Type	departmental bulletin paper
File Information	14(4)_P576-588.pdf



Cytological Studies of Tumors, XXXVII. Chromosome Conditions of Animal Tumors after Deep Freezing^{1),2)}

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(With 16 Text-figures)

During the past several years, the methods and techniques of cell preservation at low temperatures have been remarkably advanced. The feasibility of storage of various tissue cells for comparatively long periods in a frozen state has been discussed by many investigators in different specimens, such as cells in tissue culture (Scherer and Hoogasian 1954, Swim *et al.* 1958, Craven 1960), normal tissues (Blumenthal *et al.* 1950, Smith 1952), tumors (Auler 1932, Herzog 1940, Blumenthal *et al.* 1950, Bittner and Imagawa 1950, Burmester 1950, Craigie 1954, Kaziwara 1954, Morgan *et al.* 1956, Allam *et al.* 1958, Swim *et al.* 1958, Ising 1960, Sugiura 1961), bone marrow specimens (Porter and Murray 1958), and spermatozoa (Polge *et al.* 1949).

Some workers described the manifestation of new properties of tumor cells after storage at low temperatures under various experimental conditions. Morgan *et al.* (1956) and Frohberg and Matthies (1955) reported that the tumor immunity or tumor specificity was lost after deep freezing. Kaziwara (1954) observed the derivation of polyploid cell lines from the hyper diploid stock Ehrlich ascites tumor after storage at 4°C for 7 to 14 days. In the Yoshida sarcoma, Sasaki (1958) found that, in almost all cases, tumor cells of the cold storage line possessed a chromosome pattern quite similar to that of the stock tumor, excepting one tumor in which the stem-line chromosome pattern was dissimilar to that of the stock tumor. Some investigators have failed, however, to detect any change in the property of tissue cells after deep freezing (Auler 1932, Herzog 1940, Bittner and Imagawa 1950, Hauschka and Mitchell 1957, Allam *et al.* 1958, Hauschka and Levan 1958, Ising 1960).

The present investigation was undertaken in a hope to learn whether the individuality of chromosomes of the tumor stem-cells is maintained or altered following storage at low temperatures.

1) Contribution No. 521 from the Zoological Institute, Faculty of Science, Hokkaido University, Sapporo, Japan.

2) Supported by a grant to S. Makino from the Damon Runyon Memorial Fund for Cancer Research (DRG-563A) and further supported by a grant-in-aid for Fundamental Scientific Research from the Ministry of Education (No. 0434 in 1961).

The authors tender their cordial thanks to Professor Sajiro Makino for his kind direction and improvement of the manuscript. They are also indebted to Drs. Motomichi Sasaki and Takao Matsumoto for their important advice and criticism. Further, their hearty gratitude is due to Dr. Akira Sakai, Institute of Low Temperature Science, Hokkaido University, for his kind help in freezing the specimens with appropriate suggestions.

Materials and methods: The tumors employed in the present study were the MTK-sarcoma III, an ascites sarcoma of rat, and the CBA tumor-I, a mammary adenocarcinoma of mouse. The MTK-sarcoma III was artificially produced originally in the peritoneal cavity of an inbred Wistar rat in January, 1952, with the administration of *p*-dimethylaminoazobenzene (Umetani 1953): it has been serially maintained in this laboratory for over 700 transplant generations. When inoculated into the peritoneal cavity of Wistar rats, the tumor grows rapidly and causes the death of the hosts within 9 days. The CBA tumor-I is one of the spontaneous mammary adenocarcinomas originally developed in an inbred mouse of CBA strain (Kikuchi 1960): it has been successively transferred during about four years since its origin in April, 1958 for over 50 generations. This tumor is specifically transplanted into the CBA mice. After inoculating of the tumor subcutaneously, the hosts die in 29 to 30 days.

The procedure of freezing and storage of the tumors was as follows: On the 4th day after transplantation, the MTK-sarcoma III was drawn out of the peritoneal cavity of tumor-bearing rats by means of a glass capillary and dispensed in 1.0 ml quantities in 18 ampoules, nine of which contained 1.0 ml of 20% glycerol. On the other hand, the tumor tissue of the CBA tumor-I was removed from tumor-bearing mice 25 days after transplantation, minced into small pieces in physiological saline, and then transferred into 8 ampoules, half of which contained 1.0 ml of 20% glycerol. Glass ampoules, 2 cc in capacity, were used for storage. Tumor suspensions thus prepared were stored with the help of dry ice or liquid nitrogen at -30°C , -70°C and -196°C for 1, 4 and 12 weeks. After storage for the mentioned periods, 6 ampoules (3 with glycerol) containing the MTK-sarcoma III were thawed rapidly by plunging them in a water bath at 37°C . The tumor specimens were immediately used for transplantation and for counting of surviving cells. At the same time, 4 ampoules (2 with glycerol) containing the CBA tumor-I were thawed in the same way from -30°C and -196°C , and then the tumor specimens were used for transplantation. Prior to the transfer to animals, tumor materials suspended in glycerol were washed two or three times with physiological saline.

The preparations for cytological study were made according to the water pretreatment squash method with acetic dahlia (Makino 1957). The percentage of non-viable cells was calculated by the eosin method after Schrek (1936).

Observations were carried out with samples derived from 4 transfer generations after cold storage and the results were compared between before and after freezing samples.

Results

Viability and growth of frozen tumor cells: Immediately after the freezing of 1, 4 and 12 weeks, the MTK-sarcoma III cells were employed for counting of survival cells according to the eosin method of Schrek (1936). As is shown in Figure 1 the storage at -196°C seemed to be satisfactory for low-temperature preservation. On the contrary, the storage at -30°C appeared unsatisfactory, since living cells decreased in number with an increase of shrunken and damaged

cells. Generally, the storage at low temperature with glycerol seemed to be favorable for the viability of tumor cells, so far as the scope of the present experiments is concerned.

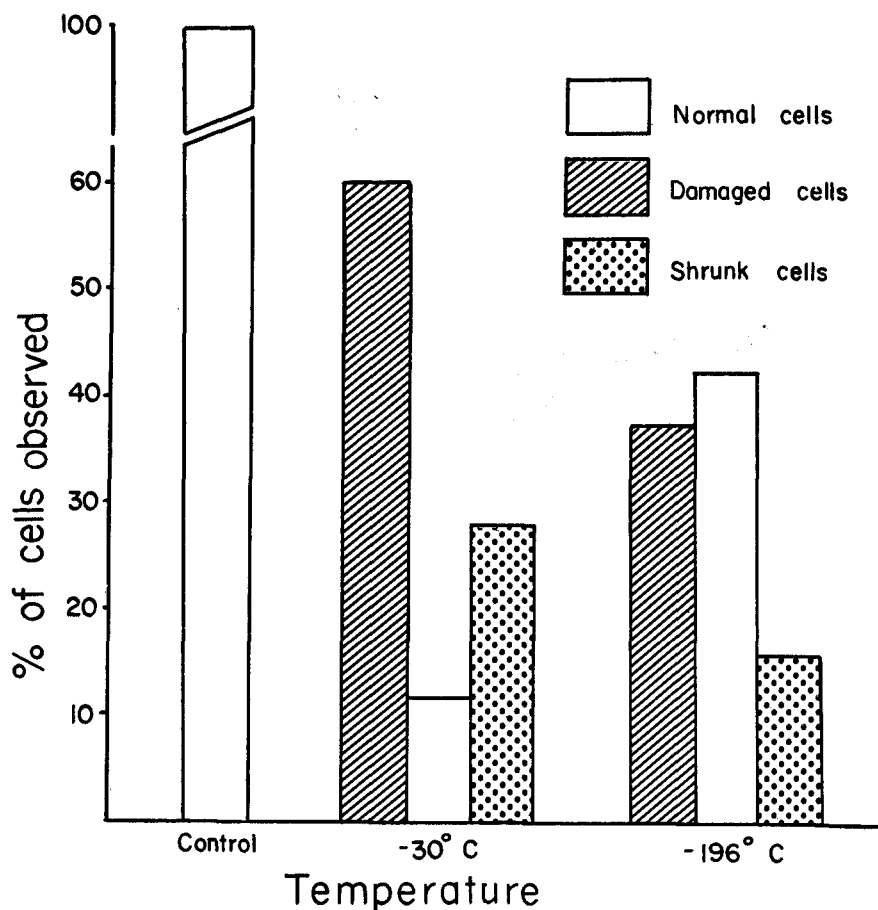


Fig. 1. Histograms showing frequencies of viable cells of the MTK-sarcoma III, before and after 12 weeks' storage at -30°C and -196°C (vital staining, eosin).

Growth rate of the frozen MTK-sarcoma III cells was recorded daily for successive days during four transfer generations. Figure 2 shows growth patterns in the first generation after 12 weeks' storage at -30°C . In normal state the growth rate of the MTK-sarcoma III continues to ascend steadily until 3 to 4 days after transplantation, then a gradual decline follows. Growth rate of the tumor cells stored at -30°C with glycerol showed a pattern similar to that of control, although

a growth activity somewhat lower than that of control was observed. On the other hand, the samples stored at -30°C without glycerol showed a markedly lower growth rate. Without glycerol, the growth rate of tumor cells after storage at -70°C and -196°C showed patterns similar to those stored with glycerol at -30°C . Cell growth recovered to the normal rate in the 2nd, 3rd and 4th generations: growth patterns of the tumors in the above three generations were nearly approximate to those observed before freezing.

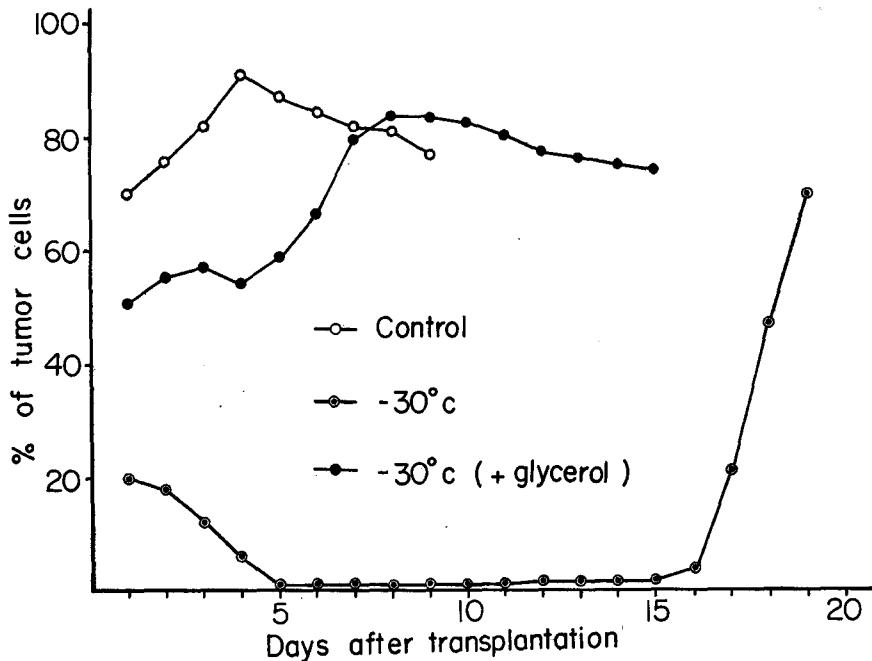


Fig. 2. Curves showing growth rate of the MTK-sarcoma III, before and after 12 weeks' storage at -30°C .

Transplantability and survival time of the tumors: Transfer experiments with the MTK-sarcoma III have shown 100 per cent lethal tumor takes to Wistar rats. In the present experiments with stored tumors, all animals which received injections of the thawed MTK-sarcoma III also showed 100 per cent tumor takes. In the case of CBA tumor-I, however, the tumors stored at -30°C without glycerol for 12 weeks were not transplantable to CBA mice, while the tumors stored at -30°C with glycerol and at -196°C with or without glycerol showed a 100 per cent lethal transplantability.

Under untreated conditions, the life span of rats bearing the MTK-sarcoma III is 8.9 days on the average, and is 29.5 days in CBA mice bearing the CBA

tumor-I. In the experimental groups, the life span of animals receiving injection of the thawed tumors was longer than that before freezing. Figure 3 illustrates the results of experiments at -196°C . The animals injected with the thawed tumor specimens prolonged the days of their life to two or three times as many as in case of use of injection before freezing, according to the duration of storing: after

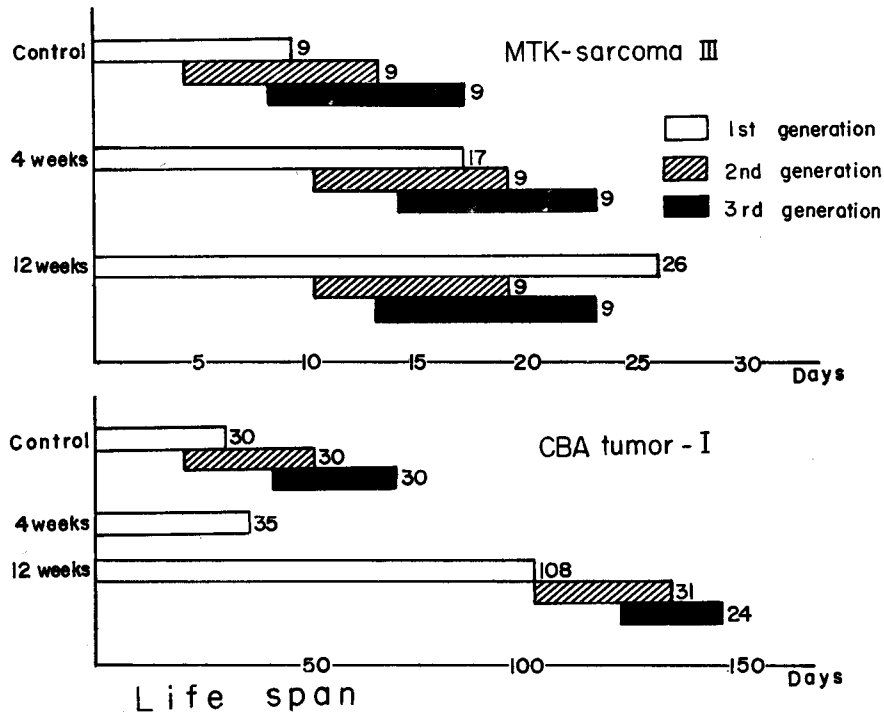
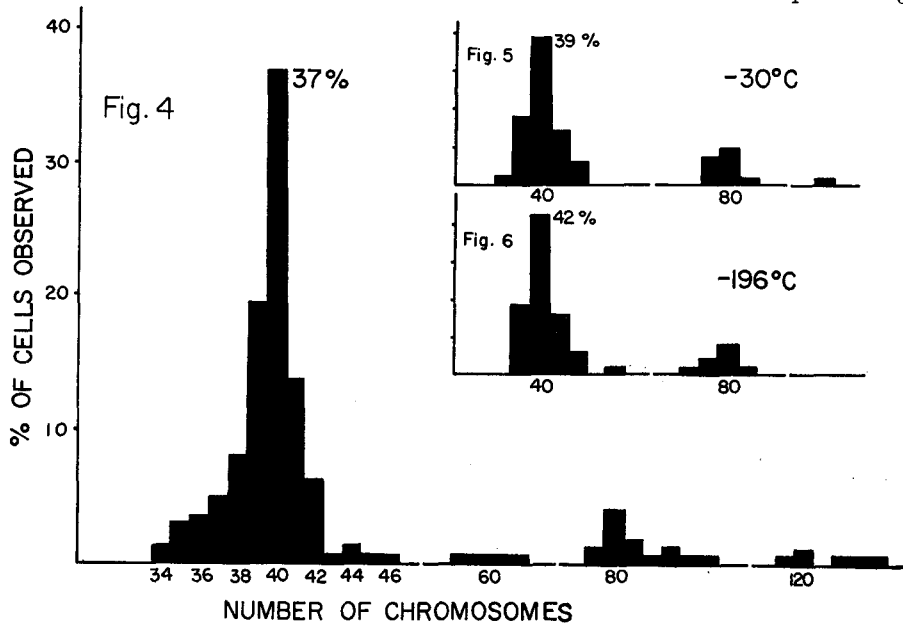


Fig. 3. Survival days of animals bearing tumors, the MTK-sarcoma III and the CBA tumor-I, after storage at -196°C .

4 weeks' storage at -196°C , the mean life span of the MTK-sarcoma III rats was 17.0 days, while after 12 weeks' storage at -196°C , it was 26.0 days. Likewise, the CBA mice bearing the CBA tumor-I showed 35.0 days in the case of 4 weeks' storage, while 108.0 days in 12 weeks' storage at -196°C . In the subsequent passages, however, they approximated those of control.

Chromosome number: The chromosome counting was made exclusively in clearly delineated reliable metaphasic cells in samples derived from frozen tumors. Though the number of chromosomes of the stock MTK-sarcoma III varies rather widely, the modal numbers lie in the diploid range (93%) with a mode at 40 (37%) (Fig. 4). The CBA tumor-I shows a chromosome variation ranging from 38 to 84, with a modal number at 40 (69%), most cells lying within the diploid range (79%)

(Fig. 7). Frozen samples of the MTK-sarcoma III showed no marked alteration in chromosome-number distribution, except for the fact that a slight increase of cells in the tetraploid range was observed in the first transfer generation (Figs. 5 and 6). On the other hand, a remarkable change of chromosome-number distribution was found to occur in the frozen CBA tumor-I: the cells in the tetraploid range,



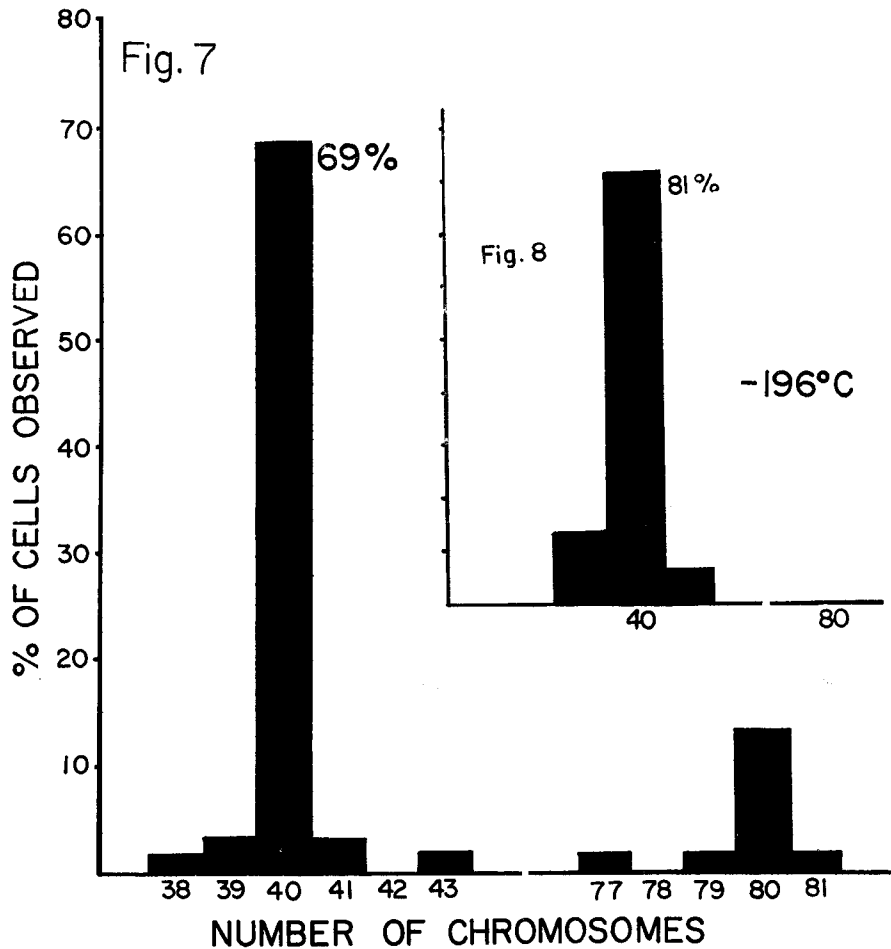
Figs. 4-6. Chromosome-number distribution in the MTK-sarcoma III cells. 4, stock tumor. 5, frozen for 12 weeks at -30°C . 6, frozen for 12 weeks at -196°C .

though they occurred in the stock tumor at about 20 per cent, disappeared, and almost all metaphasic cells under examination were diploid. In the subsequent passages, the condition returned to the level before freezing (Table 1).

Chromosome morphology: Based on the ideogram analysis of stem-line chromosomes, the individuality of the chromosomes was studied. The stem-cells of the stock MTK-sarcoma III are characterized by 8 metacentric chromosomes, 13 submetacentrics and 19 acrocentrics (Fig. 13). The stem-cells of the stock CBA tumor-I are remarkable for having 40 acrocentric chromosomes (Fig. 15) (Kikuchi 1960). Ideogram analysis has revealed that the individuality of the chromosomes of frozen tumor cells has remained unaltered in the two tumors here under consideration, since the individual chromosomes were entirely similar to those of the tumor samples before and after freezing (Figs. 14 and 16), so far as the superficial features of chromosomes are concerned.

The lengths and armratios of two marker chromosomes of stem-cells of the

two tumors were measured on the basis of 10 to 25 metaphasic cells. In the MTK-sarcoma III, the longest one and the largest metacentric one are referred to as marker I and marker II. In the CBA tumor-I, the longest one and the shortest one

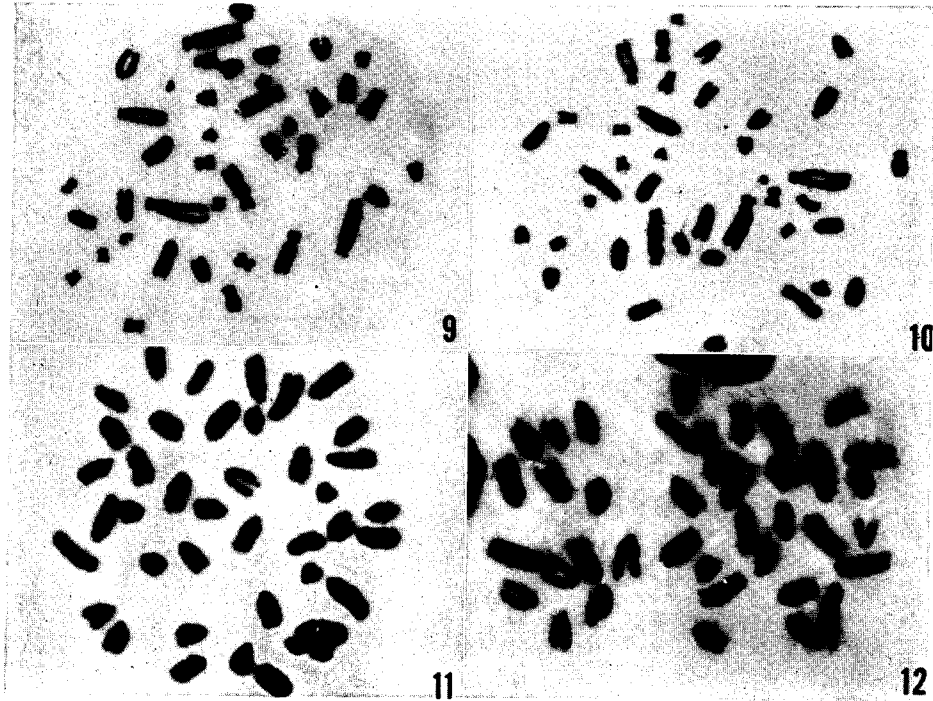


Figs. 7-8. Chromosome-number distribution in the CBA tumor-I cells. 7, stock tumor. 8, frozen for 12 weeks at -196°C .

are designated as marker chromosomes (Figs. 13 and 15, indicated by arrows). The results of measurement are as shown in Table 2; there is no appreciable change in marker chromosomes between the frozen and stock tumors.

Based on the above findings, the conclusion may be reasonable that deep

freezing of the tumors has yielded no alteration in their chromosomes, so far as their number and morphology are concerned.



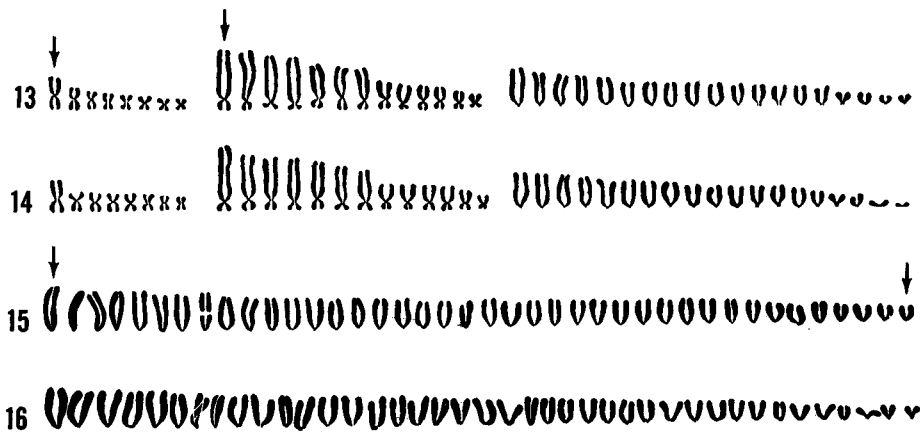
Figs. 9-12. Metaphase plates of stem-line tumor cells. 9, stock MTK-sarcoma III, $\times 1000$. 10, frozen MTK-sarcoma III (-30°C), $\times 1000$. 11, stock CBA tumor-I, $\times 1500$. 12, frozen CBA tumor-I (-196°C), $\times 1500$.

Table 1. Frequency distribution of diploid cells in the MTK-sarcoma III and in the CBA tumor-I, before and after freezing based on rough chromosome counting

Tumor	Temperature	Transplant generation			
		1	2	3	4
MTK-sarcoma III	Control	93(%)	—	—	—
	-30°C	80	85	87	90
	-196°C	84	88	93	93
CBA tumor-I	Control	79	84	72	77
	-30°C	99	93	95	86
	-196°C	100	98	85	80

Table 2. Arm ratios of marker chromosomes I and II of the MTK-sarcoma III, and ratios of length of marker chromosomes I and II of the MTK-sarcoma III and of the CBA tumor-I, before and after freezing

Material		Control	-30°C	-70°C	-196°C
MTK-sarcoma III	Arm ratio of marker I	4.9	5.1	4.9	4.7
	Arm ratio of marker II	1.3	1.3	1.1	1.2
	Ratio of marker I and marker II	1.9	2.0	2.0	1.8
CBA tumor-I	Ratio of marker I and marker II	2.7	2.6	—	2.9



Figs. 13-16. Ideogram analysis of the stem-line chromosomes in the MTK-sarcoma III and the CBA tumor-I, before and after 12 weeks' storage at -196°C (arrows indicate marker chromosomes). 13, stock MTK-sarcoma III. 14, frozen MTK-sarcoma III. 15, stock CBA tumor-I. 16, frozen CBA tumor-I.

Discussion

Studies of cell preservation at low temperatures have recently been carried out by a number of investigators with improved techniques under various experimental conditions. There are however only a few authors who have dealt with the morphological aspect of the chromosomes of frozen tumors. Morgan *et al.* (1956), Hauschka and Mitchell (1957), Hauschka and Levan (1958), and Ising (1960) ascertained that no change occurred in the chromosome pattern of tumor cells after cold storage. Kaziwara (1954) and Sasaki (1958) reported independently that a different tumor line clearly distinguishable chromosomally from the stock tumor was derived from some frozen tumors.

The results of the present study have indicated that the chromosome individuality of stem-cells showed no appreciable difference between the frozen and untreated tumors. The stability and individuality of stem-line chromosomes of tumor cells have been clearly recognized by Makino and his co-workers in a series of studies (Makino 1951a, b, Nakahara 1952, Makino and Tanaka 1953a, b, Tonomura 1953, Makino and Tonomura 1955, Tanaka *et al.* 1955, Sasaki 1956, 1958). The present findings resulted in supplementing the results of Makino and his collaborators. It should, however, be considered that the present observations dealt with only a few transfer generations. Recently Makino (1957), and Makino and Sasaki (1958) have expressed the view that the stability of the stem-line is not necessarily permanent, and can alter during serial transfer generations. It is highly probable that cold storage may induce change of the stem-line chromosomes of tumors in the course of many transfer generations with a shift of tumor properties.

In almost all cases here considered, suppression of tumor growth and prolongation of the tumor-bearing animals' life were observed in only the first transfer generation following the cold storage. This is probably due to damage of most tumor cells during storage at low temperature. Similar features were demonstrated in the cytological studies of ascites tumors following the application of chemicals by Makino and his co-workers (Makino and Tanaka 1953a, b, Tanaka *et al.* 1955, Sasaki 1956, Makino 1957, 1959, Awa 1958, 1959, Hisada 1959, Kobayashi 1960). It is interesting to see that both viability and transplantability of tumor cells were lowest in the tumors stored at -30°C without glycerol. The MTK-sarcoma III stored at -30°C without glycerol showed the highest incidence of non-viable cells and the lowest growth rate. Further, no tumor growth was observed in the CBA tumor-I under the same treatment. The storage at -30°C without glycerol seems, therefore, to be unfavorable for long term preservation of tumors. According to Porter and Murray (1958), 'thermal shock' or something similar occurs between 0°C and -79°C . This view is interesting in connection with the interpretation of the above feature. Most cells preserved at -30°C would be highly injured through the thermal shock. Porter and Murray (1958), Swim *et al.* (1958) and Craven (1960) have pointed out the necessity of glycerol in the medium. Craigie (1954) expressed the view that cancer cells exposed to extremely low temperature may be grossly damaged in three ways: 1) by pressure, 2) by penetration of the cell membrane by ice crystals, and 3) by exposure to hypertonic concentration of salts present in the intercellular fluid. With the help of glycerol, Swim *et al.* (1958) succeeded in storing cells at -70°C for three years. Hauschka and Mitchell (1957) established a frozen tumor bank and succeeded in preserving tumor cells at -76°C with glycerol. The present experiments have shown that the tumors can be preserved and remain viable at -30°C with glycerol.

In the light of the above findings, it may be possible to conclude that glycerol protects the tumors from suffering from unfavorable factors as suggested by Craigie

(1954), even though the tumor was exposed to low temperatures within the 'thermal shock' range.

The present experiments have indicated that cold storage at -70°C without glycerol resulted in a good viability of tumor cells. Craven (1960) showed that the cells stored at -70°C in glycerol survived for at least 6 weeks, whereas those stored at -20°C with glycerol failed to grow after 2 weeks' storage. Hauschka and Mitchell (1957) stored some ascites tumors for 51 months at -30°C to -60°C without glycerol. These features seem to indicate that the success of long-term cold storage is not only due to the storing temperature and the use of glycerol, but also is attributable to some other factors, unknown in nature at present.

The MTK-sarcoma III under cold storage always showed a high transplantability with 100 per cent tumor takes to Wistar rats. On the other hand, the CBA tumor-I lost its transplantability after 12 weeks' storage at -30°C without glycerol. The difference in result may be attributable to the difference of tumor specificity as well as of the method applied. In this experiment, the MTK-sarcoma III was stored in its original state, while the CBA tumor-I was minced and suspended in physiological saline. The tumor specificity to cold storage has been discussed by some workers (Bittner and Imagawa 1950, Craigie 1954). Craigie (1954) is of opinion that different types of tumors and the method of freezing exert possible effects on physiological changes during the storage at low temperatures. Swim *et al.* (1958) have also mentioned that the conditions for storage are dependent on the method of freezing and thawing, as well as on the concentration of glycerol. In conclusion, it may be important and significant to pay a good deal of attention to the method of cold storage in future experiments.

Summary

The behavior of tumor cells under storage at extremely low temperatures was cytologically investigated using the MTK-sarcoma III, an ascites tumor of rats, and the CBA tumor-I, a mammary adenocarcinoma of mice, as material. The tumors were dispensed in glass ampoules with or without glycerol, and stored at -30°C , -70°C and -196°C for 1 to 12 weeks, use being made of liquid nitrogen or dry ice.

Though not very remarkable, a considerable suppression of tumor growth and prolongation of tumor-bearing animals' life were observed in the first transfer generation of tumors after cold storage. Especially the tumors stored at -30°C without glycerol showed a marked delay of growth rate. Recovery of the growth rate of frozen tumor cells and of the life span of tumor-bearing animals to normal occurred in the subsequent passages of tumors.

It has been shown that the number as well as the morphology of the chromosomes of tumor stem-cells undergo no visible alteration as a result of exposure to extremely low temperatures, so far as the scope of the present study is concerned.

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