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**Cytological Studies of Tumors. XLV. A Chromosome
Study in Lines of MTK-sarcoma III Produced
by the Inoculation of Rapidly
Frozen Tumor Cells^{1),2)}**

By

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(With 5 Text-figures)

A bulk of information has been collected on the effect of low temperature on tumor cells (Auler, 1932; Herzog, 1940; Blumenthal *et al.* 1950; Bittner and Imagawa, 1950; Burmester, 1950; Walsh *et al.* 1950; Hodapp *et al.* 1952; Cragie, 1954; Kaziwara, 1954; Morgan *et al.* 1956; Allam *et al.* 1958; Swim *et al.* 1958; Ising, 1960; Sugiura, 1961). The present author's interest is inherent in the stability and individuality of stemline chromosomes of tumor cells following the exposure to low temperatures. Morgan *et al.* (1956) and Kimura and Kikuchi (1961) observed no visible change in the stemline chromosomes of rat ascites tumors after the low temperature treatment, while certain chromosomal alterations were reported by Kaziwara (1954) and Sasaki (1958). In the above cases, the type of cell freezing is invariably assumed to be extracellular, since the applied rate of cooling is not very rapid. Recently, it has been shown by Asahina *et al.* (1967) that some tumor cells can survive at intracellular freezing when the cooling and following rewarming in the cell is extremely rapid. They observed that very rapidly frozen cells were as transparent as intact unfrozen cells at about -30°C . This led the authors to the assumption that if intracellular ice crystals were too small to be observed under an ordinary microscope, they were innocuous to the cells in limited temperature range (Asahina, Hisada and Emura, 1967).

A change in chromosome structure may be expected during such a innocuous intracellular freezing. The present study was therefore undertaken with special concern to the karyotype stability of stemline cells of MTK-sarcoma III which were frozen and thawed very rapidly.

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Material and Methods: MTK-sarcoma III, a transplantable ascites tumor of the rat used for this experiment was artificially produced in the peritoneal cavity of an inbred Wistar rat in January 1952 with the administration of *P*-dimethylaminoazobenzene (Umetani, 1953). The tumor has been maintained in this laboratory for over 1000 transfer generations. The rats used for tumor transfer were of inbred Wistar strain, 60 to 80 grams in weight.

On the 3rd day after the tumor transfer, the tumor ascites was drawn with a glass capillary. A small amount of the ascites, about 0.002 ml in volume, was placed on a coverslip to form a thin layer. The coverslip thus prepared was rapidly dipped in liquid nitrogen for one minute. Then the ascites was observed to distinguish the type of cell freezing under a refrigerated microscope kept at about -30°C . The tumor cells were detached from the coverslip by rapidly thawing them in warm physiological saline at about 38°C . Then, the saline containing 20 drops which were separately frozen in the same manner was centrifuged at 1000 r.p.m. for 5 minutes, and the tumor cells thus collected were inoculated into peritoneal cavities of five rats. Procedures of the freezing and thawing tumor cells are referred to in the paper by Asahina *et al.* (1967).

For study of the chromosomes, the cells of each sample were treated with a hypotonic solution, and fixed with acetic alcohol. Slides were made according to the air-dry technique (Moorhead *et al.*) with Giemsa staining. The number of tumor cells studied were over 300 metaphase cells for the chromosome number, over 20 cells for the karyotype analysis.

Observations were made on samples derived from freezing lines, and the results obtained were compared with those from the stock line of MTK-sarcoma III.

Results

All the five animals received the inoculation of frozen and thawed MTK-sarcoma III cells showed 100 per cent lethal tumor takes. In the first generation, growth rate of the treated tumor cells was markedly lower than that of the control series. This is evident by a delayed appearance of mitotic cells on the 6th to 7th days after inoculation.

Three of the five rats were scanned for the chromosomal alteration. Lines of serial transfers in these three rats are tentatively referred to as designated as lines A, B, and C.

Chromosome number: The modal number of the stock line was found to be in the diploid range at 98 per cent showing a mode of 40 in cells of 85 per cent. The frequencies of cells lying in the diploid range are almost similar in the three lines A, B and C as well as in the stock line. The modal number at 40 occurred in the samples from A and B lines except C line. The chromosomes of the latter line were in a similar diploid range, 97 per cent in the 2nd and 98.5 per cent in the 4th transfer generations. The modal number of the C line was 39. The difference in the C line tended to gradually decrease in the later transfer generations. A similar tendency was observed in later transfer generations of the stock tumor.

Chromosome morphology: Based on the karyotype analysis of the stemline chromosomes, the chromosome individuality was investigated in the stock and frozen lines. It was reported by Umetani (1953) in the MTK-sarcoma III that the longest chromosome was metacentric in 32.7 per cent of cells, while it was submetacentric in the 67.3 per cent of cells. The occurrence of the two types of cells was confirmed

again in the present study. It was found that almost all the 40 chromosome-cells had the longest chromosome of a submetacentric nature, while the longest one was represented by a metacentric element in the 39 chromosome-cells. The comparison of the two kinds of cells revealed that the 40 chromosome-cell was characterized by 10 metacentric chromosome, 13 submetacentrics of which the 10th element bore a distinct satellite on its short arm, and 17 acrocentrics of which the 13th one was also satellited (Fig. 2), and that the 39 chromosome-cell comprised 11 metacentric chromosomes, 13 submetacentrics of which the 10th chromosome showed a prominent satellite on its short arm, and 15 acrocentrics of which the 11 element was also satellited (Fig. 4). Similarly, the two types of cells corresponding to the 40- and 39- cells of the stock line were observed in A, B and C lines. Karyotype analysis of the 40- cells revealed that the chromosome constitutions occurring in the A, B and C lines were consistently similar to that of the stock line (Fig. 3), and further that the chromosomes of the 39-cells (Fig. 5) showed no morphological alteration between the untreated and treated samples.

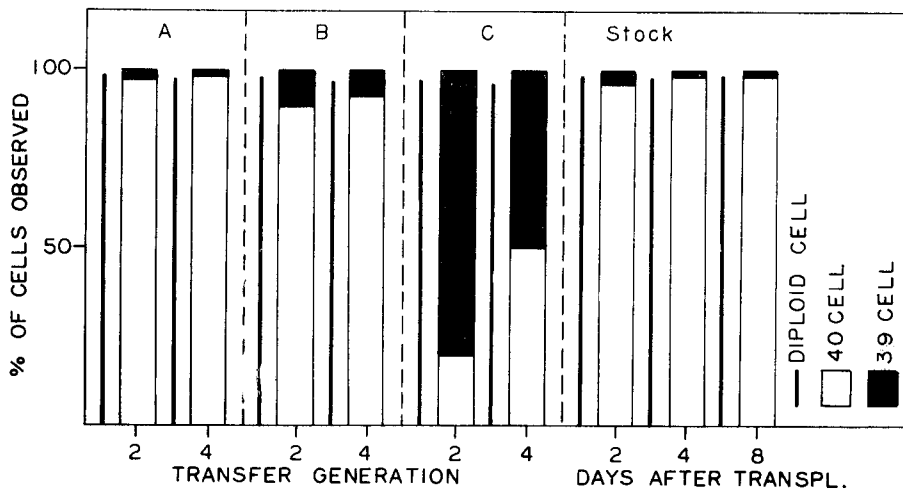
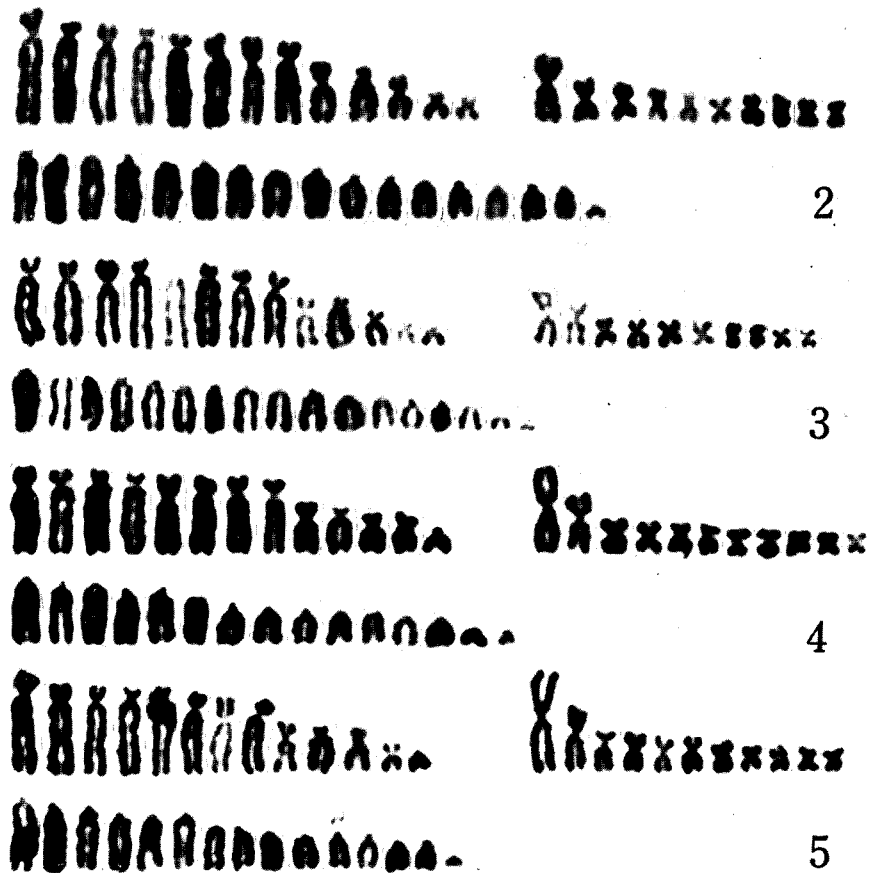


Fig. 1. Frequency distribution of 40 chromosome-cell and 39 chromosome-cell in the MTK-sarcoma III stock line and rapid freezing lines A, B and C. For detail, see text.

The results of the above investigations seem to imply that the chromosomes of the tumors produced by the inoculation of tumor cells which have been frozen and thawed very rapidly, undergo no alteration numerically and morphologically following the treatment.

Discussion

While considerable reports have lately been available on cell freezing experi-



Figs. 2-5. Karyotype analyses of 40-cells and 39-cells in the MTK-sarcoma III stock line and rapid freezing lines. 2 and 4, from stock tumor. 2; 40 chromosome-cell. 4; 39 chromosome-cell. 3 and 5, from freezing lines. 3; 40 chromosome-cell. 5; 39 chromosome-cell.

ments in various organisms, there are a few authors who have dealt with morphological aspects of the chromosomes in frozen tumors. Morgan *et al.* (1956), Ising (1960), Kimura and Kikuchi (1961) showed that no change occurred in the chromosome pattern of tumor cells after cold storage, whereas Kaziwara (1954) and Sasaki (1958) reported independently that a different tumor line with a chromosomal change was derived from some frozen tumors.

The results of the present study have indicated that the chromosome constitution of stem-cells undergoes no alteration by means of rapid freezing, and that the stemline chromosomes of the stock tumor have remained unchanged in the tumor

lines produced by the inoculation of tumor cells which have been frozen very rapidly. The stock tumor was characterized by two stemlines having 40 and 39 chromosomes, respectively. In freezing lines, A and B, the frequency of the 40-cells was approximately the same, being higher than 90 per cent, while in line C, the 39-cells were predominant, showing 75 per cent in frequency. Sasaki (1960) reported that 4V-type cells of subline D of Yoshida sarcoma were generally overcome by 3V-type cells of subline C in the mixed cell population under the influence of selective pressure. It is likely that the 40-cells are less dominant in the population of the line C than in those of the two other lines. The increase in number of the 40-cells observed in the 4th transfer generation of this line can be explained as thus: after certain transfers, the predominancy of the 40-cells might be established.

According to Umetani (1953), the chromosome number of the stem cells of the MTK-sarcoma III varied rather widely having a mode at 40 (13%), while Kimura and Kikuchi (1961) reported that the chromosome number of the MTK-sarcoma III showed a wide variation with the same mode at 40 in higher incidence (37%). The results of the present study (1966) are as shown in Figure 1: the cells lying in the diploid range were 98 per cent in frequency with a modal number of 40 in 85 per cent. An increase in frequency of the modal cells may be attributable to the fact that the nucleoplasmic balance in relation to the activity of the tumor cell has increased with successive transfer generations.

The present study detected the difference of the chromosome constitution between cells having 40 chromosomes and those containing 39 chromosomes as follows: the 40-cells consisted of 10 metacentrics, 13 submetacentrics and 17 telocentrics, whereas the 39-cells comprised 11 metacentrics, 13 submetacentrics and 15 telocentrics. It is of some interest to mention that in the stock line, two arms of the longest metacentric chromosome of the 39-cells correspond to two telocentric probably of No. 5 to No. 9 in the 40-cells.

Asahina *et al.* (1966, 1967) have suggested that even the ice formation in protoplasm is innocuous to the tumor cells when the intracellular ice crystals are very small. The results of the present study are sufficient to indicate that the rapidly frozen-thawed tumor cells can remain without damage and maintain their stemline characteristics. For the final conclusion, further experiments under various freezing and thawing conditions are needed in future.

Summary

The chromosome condition was studied in lines produced by the inoculation of tumor cells of the MTK-sarcoma III which received rapid freezing at and thawing from liquid nitrogen temperature.

Evidence was presented that the stemline chromosome of tumor cells of rapidly frozen-thawed line showed no visible alteration, being numerically and morphologically similar to those of the stock line.

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