Experimental Studies on Application of Hypothermia to Cancer Chemotherapy

by

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Received for Publication May 4, 1964

INTRODUCTION

Since Gilman and Phyllips[14] found a tumor-inhibiting action in nitrogen mustard in 1946, a great number of anticancer chemotherapeutics has been developed. Although these drugs are an available adjunct to cancer therapy, they are not curative agents and still involve many problems, the most important of which are their toxic side effects. While the search for a newer and more effective anticancer agent has proceeded, many efforts have so far been made to increase the efficiency of the currently available anticancer agents, especially for the purpose of reducing their side effects. These attempts have proceeded in a number of ways. Some investigators have been trying to intensify the effects of the drugs by increasing their local concentration. KLOPP et al. (1950)[8] devised a method of intra-arterial administration of anticancer agents and attempted to deliver larger dosage of the drugs to tumor tissue with less systemic toxicity. For the similar purpose, GREECH et al. (1958)[9] proposed regional perfusion chemotherapy. These methods may be classified as physical ones. Biological methods have also been studied to enhance the effectiveness of anticancer drugs. In our country, for example, SHIRAI[34] maintained an effective concentration of the drugs in the blood for the entire period of generation time of tumor cells and succeeded in intensifying their therapeutic effects.

TAKAHASHI[40], a member of our research group, has recently reported that when tumor-bearing mice were subjected to a certain period of hypothermia at 20°C, DNA-synthesis in tumor tissue measured by autoradiography became maximum at a certain time after rewarming the animals to normal body temperature, and that an alkylating agent administered at the time of maximum DNA synthesis resulted in a greater regression of the tumors than in the control group treated merely with the drug. These results led us to presume that after being subjected to hypothermia cancer cells can be synchronized in vivo in regard to mitosis, and that when anticancer agents are administered to the synchronous cell populations in the most sensitive phase, the effects of the agents can be intensified. The present study has been carried out to examine the presumption by use of experimental mouse tumor of solid form.

Mice which were bearing experimental solid tumors were subjected to hypothermia at 20°C for a certain period of time, and changes in mitotic counts in the tumor tissue obtained during and after hypothermia were examined. Thereafter, based on the work hypothesis mentioned above, a new method to intensify the effects of anticancer agents

was applied to an experimental chemotherapy for the solid tumors.

EXPERIMENT I : CHANGES IN MITOTIC COUNTS INDUCED BY HYPOTHERMIA

1. MATERIALS AND METHODS

1) Animals and tumors

The animals used in the present experiment were male mice of dd strain, one month of age, each weighing about 20 grams. The mice were supplied from the Central Breeding Station of Experimental Animals, Kyoto University, and fed with a standard solid diet and water given ad libitum.

Two kinds of ascites cancer were employed; Ehrlich ascites tumor which has been maintained by successive intraperitoneal transplantation to healthy carriers in our laboratory, and Sarcoma 180 obtained from the Shionogi Research Laboratory in 1961 and maintained in the same way. From the abdominal cavity of a carrier inoculated with the tumor cells 5 to 7 days previously, fully developed ascitic fluid was removed aseptically by a syringe and diluted with saline solution. About 5 million tumor cells in 0.2 cc of the diluted fluid were injected subcutaneously into healthy animals in the right axillary region. The transplantation resulted in developping subcutaneous solid tumors. The rate of successful transplantation by this method was nearly 100% in both Ehrlich carcinoma and Sarcoma 180. The subcutaneous solid tumors steadily increased in size, thus growing up as large as 1.0 to 1.5 cm in diameter on the 10th day after inoculation.

2) Hypothermia

Ten days after the inoculation the mice were subjected to hypothermia in the following way. After being injected intraperitoneally with Nembutal at a dose level of 37.5 mg/kg and Wintermin at a dose level of 15 mg/kg, the mice were placed on a tray in an ice box so that the body temperature might fall rapidly to the level of hypothermia at 20°C. The tray was then transported on cold water to maintain their body temperature at a constant level of 20°C ± 2°C. The body temperature was measured in the rectum at regular intervals with an electric thermometer.

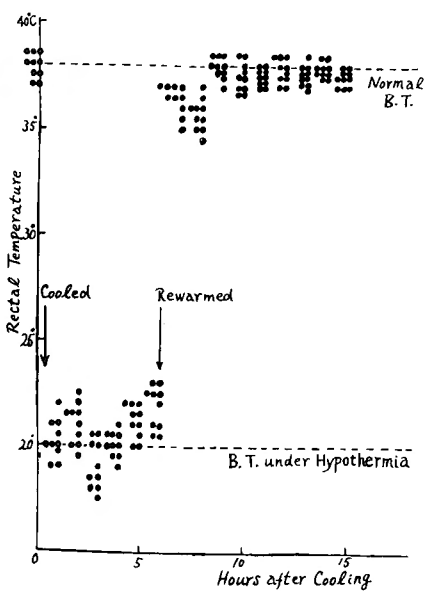

Fig. 1 Changes in rectal temperature in tumor-bearing mice subjected to 6 hours hypothermia at 20° ±2°C.

After the hypothermia for 2, 6 or 10 hours, the tray was placed on hot water to restore the body temperature of the animals to the normal level at about 38°C. The changes in the body temperature of the mice subjected to 6 hours of hypothermia at 20°C ± 2°C are presented in Fig. 1.

3) Counting of tumor cells in mitosis

The tumor-bearing mice thus subjected to hypothermia were killed at regular intervals during and after hypothermia and the subcutaneous tumors

in the axillary region were removed to make sections for histological examination. During hypothermia the tumor-bearing mice were killed at hourly intervals, and after hypothermia at intervals of two hours up to 40 hours after rewarming. From the tissue fragments of the removed tumors which were fixed in 10% Formol water, sections of 2 to 3 microns in thickness were prepared. After being stained with hematoxylin and eosin solution, the sections were used for histological examination, especially for counting tumor cells in mitosis. Control animals, anesthetized in the same way as in the experimental animals, were placed in the environment at 38°C, and were killed at intervals of two hours for the similar histological examination.

During the course of experiment, it has been made a rule that mice were paired off and always two mice were sacrificed simultaneously. Two sections were prepared from the circumferential area of each tumor, where tumor cells were the most viable. On each of the sections 2000 tumor cells were examined. The mitotic index at a certain time was given by counting the total number of 8000 tumor cells from a pair of mice.

4) Counting of cells in different stages of mitosis

The proportion of the cells in the different stages of mitosis (Pre-, meta-, ana-, and telophase) was also determined in parallel with the mitotic index.

According to KUWADA[20][21], the whole course of prophase is to be divided into six stages; ranging from early prophase in which a structure of long and slender thread-shaped chromosomes is apparent in the resting nucleus to late prophase in which a structure of spiral chromosomes is to be seen in the nucleus with the nuclear membrane no more visible.

As to prophase, counting was made only on the cells in late prophase, in which the mitotic figure could be recongnized most distinctly. In the present experiment, therefore, the cells in late prophase are denoted merely as the cells in prophase (Fig. 2).

Figs. 3 & 4 show the cells in metaphase with pairs of chromosomes standing in a row on the equator. Upon entering anaphase, as shown in Fig. 5, the pairs of chrmosomes on the equator separate from each other and remove towards the poles. In telophase (Fig. 6), re-organization of the separated daughter chromosomes are noticed and division of cytoplasma occurrs thereafter.

3. RESULTS

A) Sarcoma 180 (solid tumor)

The mice which were bearing Sarcoma 180 were subjected to hypothermia at 20°C ±2°C for 2, 6 or 10 hours.

1) Histological findings in Sarcoma 180 subjected to hypothermia

Histological findings in Sarcoma 180 solid tumor obtained from the mice of normal body temperature are presented in Fig. 7. The tumor tissue, full of round tumor cells with few blood vessels, shows a picture of round cell sarcoma. The tumor cells are isolated from each other, among which several multinucleated giant cells are seen. While necrosis and hemorrhage are occasionally found in the midst of the tumor, there are marked proliferation and infiltration of tumor cells with many mitotic figures in the periphery.

Histological changes in the tumor obtained during hypothermia were as follows.

Firstly, in case of 2 hours of hypothermia, no degenerative changes both in nuclei and in cytoplasm were observed, some cells being still in mitosis (Fig. 8). Secondly, in case of 6 hours of hypothermia, little degenerative changes were noticed. The number of cells with mitotic figures was far less than in the case of 2 hours of hypothermia (Fig. 9). Thirdly, in case of 10 hours of hypothermia, such slight degenerative changes as vacuolar degeneration of cytoplasma and condensation of nuclei were recognized in some tumor cells. There was a marked decrease in the number of cells with mitotic figures (Fig. 10).

Histological changes in the tumor obtained after the release from hypothermia were as follows. In the group subjected to 2 or 6 hours of hypothermia, there was a steady increase in the number of dividing cells shortly after rewarming and the slight degenerative changes in the tumor cells which were noticed during hypothermia diminished in process of time. (Figs. 11 & 12).

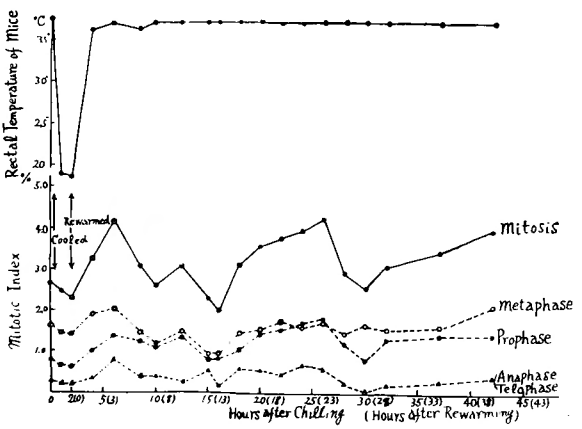

Fig. 15 Changes in mitotic index in Sarcoma 180 solid tumor from mice subjected to 2 hours hypothermia at 20°± 2°C.

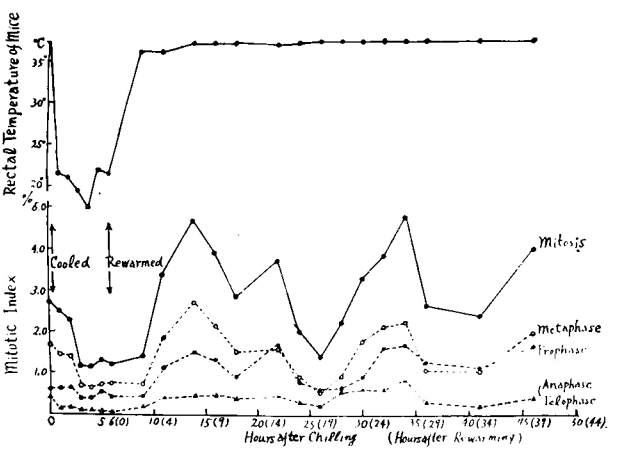

Fig. 16 Changes in mitotic index in Sarcoma 180 solid tumor from mice subjected to 6 hours hypothermia at 20° ± 2°C.

In the group subjected to 10 hours of hypothermia, the increase in the number of mitotic tumor cells after rewarming was found to be more rapid and marked than in the case of the shorter period of hypothermia. The degenerative changes in tumor cells which were found during hypothermia diminished gradually, and almost completely disappeared at the 24th hour after rewarming (Figs. 13 & 14).

2) Changes in mitotic counts in Sarcoma 180 induced by hypothermia

2-1) Group of mice subjected to 2 hours of hypothermia (Fig. 15)

In Fig. 15 are shown the changes in mitotic index in the tumors from the mice subjected to 2 hours of hypothermia. The proportion of the tumor cells in mitosis before hypothermia was approximately 2.7 , of which the proportion of the cells in metaphse was the largest, holding a value of about 1.7%. The cells in prophase followed

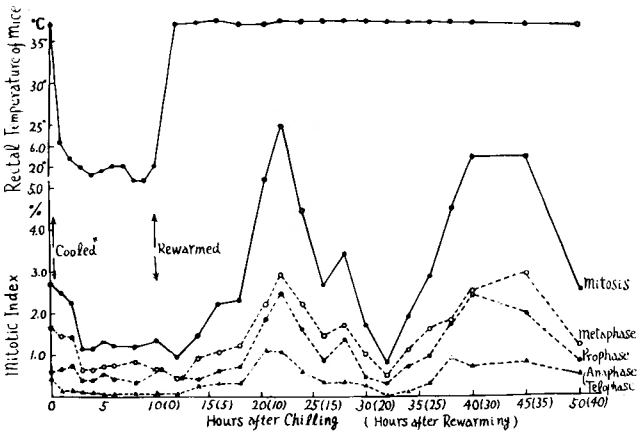

Fig. 17 Changes in mitotic index in Sarcoma 180 solid tumor from mice subjected to 10 hours hypothermia at 20°±2°C.

those in metaphase, while the proportion of the cells in anaphase-telophase being the smallest of all mitotic cells.

When the mice were cooled, the mitotic index decreased gradually, still holding a value of 2.3% 2 hours after the beginning of hypothermia. Immediately after rewarming, there was a relatively rapid increase in the mitotic index, which reached a maximum value of 4.2% 4 hours after rewarming, presenting the first peak of mitotic curve. Then the index decreased rapidly, showing a low value of 2.0% 14 hours after rewarming. After that time, there was again a steady increase in the mitotic index which, at the 24th hour after rewarming, reached almost as high a value as the first peak of mitotic index. The changes in the proportion of the cells in various phases of mitosis were almost paralleled with those in the mitotic index described above.

2-2) Group of mice subjected to 6 hours of hypothermia (Fig. 16)

Shortly after the beginning of hypothermia, there was a gradual and steady decrease in the mitotic index, the value of which then fell abruptly to a level of 1.15% at the 3rd hour of cooling. This low value remained almost at the same level during the whole period of hypothermia. After rewarming, the mitotic index remained nearly constant at a low value of less than 1.4% for a short period of time. Then it began to increase rapidly 3 hours after rewarming, to reach a maximum value of 4.7% 8 hours after rewarming. Thereafter the mitotic index decreased to a minimum value of 1.4% 20 hours after rewarming, and then increased again until at the 28th hour it achieved almost as high a value as that of the first peak. On the whole, the curve of mitotic index in the group of 6 hours of hypothermia resembles to that in the group of 2 hours of hypothermia, except that in the former group the appearence of the first peak was delayed by 4 hours and its mitotic curve thereafter was considerablly steep, as compared with the latter group. The percentage of the cells in each phase of mitosis, similarly to that in the case of 2 hours of hypothermia, changed almost in parallel with the mitotic index.

2-3) Group of mice subjected to 10 hours of hypothermia (Fig. 17)

After the commencement of hypothermia, the mitotic index decreased almost in common with that in the cases of the shorter period of hypothermia; the mitotic index declined abruptly at the 3rd hour of cooling, and thereafter remained nearly at a constant low level of approximately 1.2% throughout the whole period of cooling. During the first 2 hours after rewarming, the mitotic index seemed to decrease. Thereafter the mitotic index increased very rapidly, achieving a maximum index of 6.4% 12 hours after rewarming. Twenty-two hours after rewarming the index reduced again to a minimum of 0.8%, which was much lower than that observed during hypothermia. It is worth notice that in this group the mitotic index continued to keep the low level even during the first few hours after rewarming, and the first peak of the mitotic index appeared later with much steeper slopes than in the groups of the shorter period of hypothermia.

The proportion of the cells in the different mitotic phases changed almost in parallel with the mitotic index. It is of interest that the cells in anaphase-telophase which were not found during hypothermia increased strikingly in number after rewarming.

B) Ehrlich carcinoma (solid tumor)

The mice which were bearing Ehrlich carcinoma were subjected to 6 hours of hypothermia at 20°C.

1) Histological findings in Ehrlich carcinoma subjected to hypothermia

Histological findings in the tumors before hypothermia are presented in Fig. 18. There is a considerable number of cells with mitotic figures. The tumor cells are rich in basophile cytoplasm and have nuclei which are also abundant in chromatin. The cells vary in size, and infiltrate into the peripheral tissues. In the central area of the tumor, however, necrosis and hemorrhage are frequently observed.

During hypothermia, the number of mitotic figures decreased markedly and the cells showed slight degenerative changes as in the case of Sarcoma 180 (Fig. 19).

After rewarming, the findings of degenerative changes diminished gradually in the course of time, and a steady increase in the number of mitotic cells occurred (Fig. 20). These observations are almost similar to those in the case of Sarcoma 180.

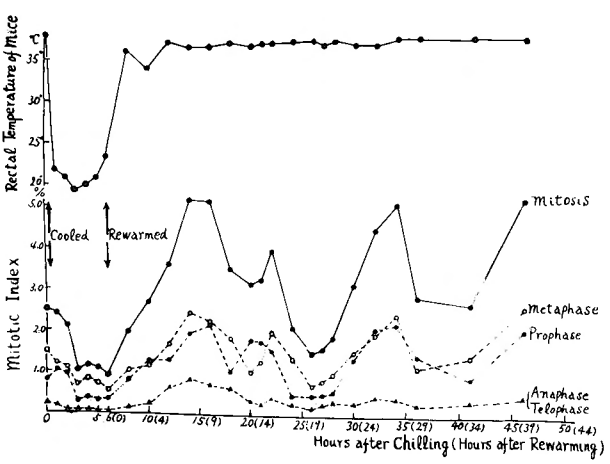

Fig. 21 Changes in mitotic index in Ehrlich solid carcinoma from mice subjected to 6 hours hypothermia at 20°±2°C.

2) Changes in mitotic index in Ehrlich carcinoma induced by hypothermia (Fig. 21)

Mitotic index in the tumors from the mice subjected to 6 hours of hypothermia was observed to change almost similarly to that in the group of Sarcoma 180 cooled for 6 hours.

As is indicated in Fig. 21, the percentage of the cells in mitosis,

which had a value of about 2.5% before cooling, decreased rapidly to a level of 1.1% at the 3rd hour of hypothermia. The index thereafter remained almost at the same low level throughout the period of hypothermia.

After rewarming, the mitotic index increased rapidly to achieve, at the 8th hour after rewarming, about twice as high a value as that in normal body temperature. After that time, there was a marked decrease in the mitotic index, until at the 20th hour the index reached a minimum value. Then it increased again to reach almost the same value as that of the first peak at the 28th hour after rewarming. The index of the cells in each phase of mitosis changed almost parallel to the mitotic index. It is of interest that during hypothermia the cells in prophase decreased most noticeablly among those in the other mitotic phases.

C) Influence of basic anesthesia on mitotic index in Sarcoma 180 (Fig. 22)

In the control group of Sarcoma 180 bearing mice, which were anesthetized in the same way as in the experimental groups, changes in the mitotic index in the tumors were examined. As is shown in Fig. 22, an increase in the mitotic index, at a level of 3.8%, was observed 2 hours after administration of the narcotics. The increase was characterized by a rise in metaphase index. Thereafter the mitotic index remained, although with slight fluctuations, almost at the same level between 2 and 3% for at least 25 hours.

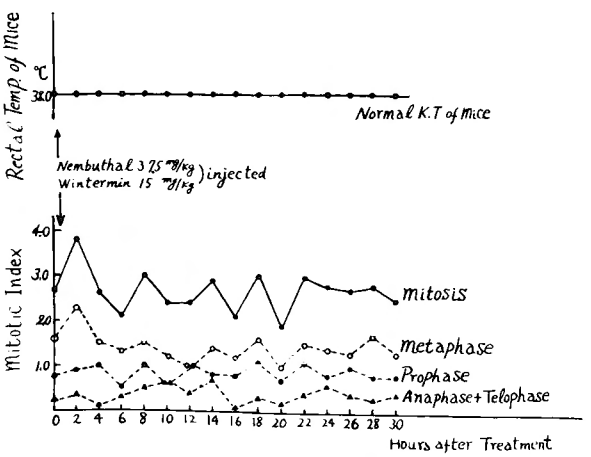

Fig. 22 Changes in mitotic index in Sarcoma 180 solid tumor after intraperitoneal injection of Nembutal 37.5 mg/kg and Wintermin 15 mg/kg, under normothermia.

3. DISCUSSION

In 1959, NEWTON and WILDY[28] exposed HeLa cells previously subcultured at 37°C to a sublethal temperature of 4°C for one hour and then replaced them at 37°C. In their experiment it was demonstrated that there were little or no cell divisions for 17 to 18 hours after chilling and then as many as 60-80% of the cells divided within one hour or less, and that such bursts of cell division were observed at intervals of 18 hours for at least two division cycles. In the previous year, SCHERBAUM and ZEUTEN[31)43)] found that when cultures of Tetrahymena pyriformis were given several periodic heat shocks,

that is to say, environmental temperature was shifted repeatedly from the optimum of 29°C to the sublethal value of 34°C, there appeared later explosive divisions of as many as 85% of the cells. The phenomenon of this kind of explosive cell divisions has been referred to "synchronization of cell division". Although the mechanism of the synchronization induced by temperature-shifts has not yet been clarified, some explanations have been proposed[24)38)]. Cells are not equally sensitive to temperature shocks at all "ages", namely, in all stages of the division cycle. Cells in any stages between "predivision period" and telophase are not or almost not sensitive to temperature shocks, despite of which the cells complete division, though the mitotic time is more or less prolonged. The predivision period is called "point of no return". The sensitivity of cells to temperature increases with "age" during the second half of interdivision period. The nearer metabolic process of cells advances to this "point of no return", the more temperature-sensitive they become, so that a given temperature shock sets these older cells back as far as to "younger stages". On the other hand, the cells in the first half of interphase are less sensitive and can advance to the sensitive stage to be finally caught by the temperature shock. Thus most of the cells are placed almost in the same stage of the division cycle, resulting in synchronous cell division after their return to normal temperature.

Since the studies by SCHERBAUM et al. and NEWTON et. al. numerous in-vitro experiments on temperature-induced synchronous division[6)7)15)17)22)33)] have been reported. In regard to in-vivo synchronization of cell division, however, little information has been available. Recently, TAKAHASHI[49)] examined influence of hypothermia upon the metabolism of nucleic acid in NF-Sarcoma cells by P^{32} autoradiography and demonstrated that when tumor-bearing mice subjected to hypothermia at 20°C for 6 hours were returned to normal body temperature, the rate of incorporation of P^{32} into DNA became maximum in the period between 2 to 4 hours after rewarming. These results led us to have a presumption that some degree of in-vivo synchronization in mitosis might be induced by a cold shock. The present author has attempted to apply the principle of in-vito synchronization of mitosis to tumor cells which are growing in experimental animals and has examined whether any degree of synchronous mitosis in in-vivo tumor cells could be induced by a cold shock.

The present study began with an observation upon changes in the mitotic index during hypothermia. While in case of 2 hours of hypothermia there was not a remarkable decrease in the mitotic index, the index decreased markedly at the 3rd hour of hypothermia in the cases of both 6 and 10 hours of hypothermia. T. Fay[13)] reported that, in human cancer, no mitotic cell was found when the body temperature was lowered below 32°C. However, according to EVANS et al[11)]., although the duration of both division time and generation time in Vicia faba cells was prolonged in accordance with decrease in culture temperature, the cell division was not completely blocked at as low a level as 3°C. The marked decrese in the mitotic index observed at the 3rd hour of hypothermia in the present experiment might be due to the cold shock, which might be effective enough to prevent the tumor cells from entering division. The fact that after the rapid decrease of the mitotic index at the 3rd hour of hypothermia, the index remained almost at a constant low level during the rest of the period of hypothermia seems to imply that,

as stated by Evans, the division time of tumor cells is remarkably prolonged during hypothermia. Moreover, in case of Sarcoma 180 treated with 10 hours of hypothermia, mitotic index was observed to decline gradually during the first several hours after rewarming, suggesting that cells actually undergoing mitosis at the time of hypothermia complete the mitotic episode on being returned to normal body temperature. MAZIA[24] reported that in tumor cells metaphase was the most sensitive to temperature shocks among all phases of mitosis, and its duration was extraordinarily prolonged as temperature decreased. On the other hand, Hunter-SZYBALSKA et al[17]. found that during the period of chilling in cultures of Bacillus megaterium almost all the cells were in the stages of metaphase and early anaphase, but upon being rewarmed, there was a marked increase in the number of cells undergoing separation of sister chromonsomes. From these observations, they reached a conclusion that the most obvious effect of chilling on divising cells was to inhibit selectively a metabolic process connected with separation of sister chromosomes, i. e. the transmission from metaphase to anaphase. Similar findings were also observed in the present experiment, in which during hypothermia the cells in metaphase were larger in number than those in other mitotic stages and the number of the cells in anaphase-telophase was extremely small. After rewarming, however, the cells in anaphase-telophase markedly increased in number as compared with those in the other mitotic stages.

Study was also made on the changes in miotic index after the release from hypothermia. It was of interest that there appeared a marked peak in the mitotic curve at the 4th, 8th and 12th hour after rewarming, respectively in the cases of 2, 6 and 10 hours of hypothermia, indicating that the longer the duration of hypothermia was, the higher was the peak. In HeLa cells, as reported by NEWTON et al[28], the interval between the rewarming and the first burst of cell division roughly corresponded to the normal interphase period, but in some cells[32] synchronized by temperature shocks, the interval was shorter than their normal interphase period. HUNTER-SZYBALASKA[17] argued that the interval depended on the degree of temperature shocks. In the present experiment, in which the body temperature of mice was maintained at a constant level of 20°C, it was observed that when the period of hypothermia was prolonged, the appearence of first peak in the mitotic curve was delayed for the time corresponding to the prolonged period of hypothermia. The fact that the maximum value of the mitotic index after rewarming increased in accordance with the prolongation of cooling period is considered to indicate that hypothermia of a longer duration brings more cells near to the "point of no return".

In the present experiment, the interval between the first and second peaks of mitotic index was found to be approximately equal to the mean generation time of these tumors, i. e. 18 to 20 hours. The fact that the second peak was found to be almost as high as the first one suggests that the same degree of synchrony was preserved for at least two division cycles after rewarming. Other reports[7][24][43] in the field of synchronous cell cultures, however, indicated that the degree of synchrony gradually decreased as time passed after rewarming, and each value of the following peaks was somewhat lower than the preceeding one.

In the control animals which were merely injected with Nembutal and Wintermin, a temporary increase in mitotic index, especially in metaphase index, was observed immediately

after the administration of the narcotics. After that time, however, the index remained almost at a constant level of the same value as observed before the treatment. According to Mc-Erloy[25], narcotics including Nembutal and Wintermin produce a colchicine-like effect of reversible protein-denaturation on cells. The increse in metaphase index observed in our control goup may be attributed to this colchicine-like effect of the narcotics.

In short, the changes in mitotic counts which were observed in the present experiment are very close to those observed in the other synchronous cell cultures[21][28][31][33], in which the actual increase in cell numbers was measured. The comparision of the findings led us to have a belief that tumor cells, when exposed to a cold shock, might be placed thereafter in a state of in-vivo synchrony for a certain period of time.

Temple Fay et al[37] found that undifferentiatiated cells in chick embryos and some human cancer which were placed at a low temperature of 32°C for 48 hours showed such reversible cytological changes as swelling of cytoplasm and a slight loss of nuclear details. When the temperature of this or lower level was continued for more than 72 hours, however, the degenerative changes in the cells became irreversible. In the present experiment, it was observed that in case of 6 or 10 hours of hypothermia the slight degenerative findings which were noticed during the hypothermia were gradually declined after rewarming and disappeared 24 hours later. Hypothermia of this degree might not produce any irreversible degenerative changes in the tumor cells.

4. SUMMARY

Three groups of mice which were bearing subcutaneous solid tumors of Sarcoma 180 were subjected to hypothermia at 20°C for 2, 6 and 10 hours respectively. The tumors were removed during and after the hypothermia to study the changes in the mitotic index as well as in the percentages of the cells in various phases of mitotic cycle. Similar examinations were also made on a group of mice with subcutaneous solid tumors of Ehrlich carcinoma, which were cooled to the same temperature for 6 hours.

The results were as follows:

1 : Immediately after the commencement of hypothermia, the mitotic index decreased gradually and then promptly fell at the 3rd hour of cooling. The mitotic index thereafter remained at a constant low level during the whole period of hypothermia.

2 : During the hypothermia, the percentage of the cells found in metaphase remained relatively high, as compared with that in the other phases of mitosis, while after rewarming there appeared a marked increase in the percentage of the cells in anaphase-telophase. This suggests that the transition stage from metaphase to anaphase is the most sensitive to low temperatures among all stages of mitosis.

3 : After rewarming, the mitotic index reached its maximum value at the 4th, 8th and 12th hour, respectively in the cases of 2, 6 and 10 hours hypothermia. The longer the duration of hypothermia was, the later appeared the first peak with a higher value of the mitotic index.

4 : The first peak of the mitotic index was followed by the second high value after approximately 20 hours. The interval between these two peaks in the mitotic index roughly corresponded to the mean generation time of the tumor cells concerned. The value of

the two peaks were found to be almost same.

5 : The slight damages of the cells which resulted from hypothermia disappeared gradually after rewarming. It was suggested that hypothermia of this degree would produce no irreversible changes in the tumor cells.

6 : These results were compared with the other observations reported in the field of synchronous cell cultures, and it was concluded that in-vivo synchronous mitosis of solid tumor cells might be introduced by subjecting tumor-bearing animals to hypothermia.

EXPERIMENT II : INTENSIFICATION OF EFFECTS OF ANTICANCER AGENTS BY USE OF HYPOTHERMIA

1. MATERIALS AND METHODS

Use was made of male mice of dd strain, one month old, each weighing about 20 gram. The mice were inoculated subcutaneously in the right axillary region with about 5 million ascitic tumor cells of either Sarcoma 180 or Ehrlich carcinoma. The inoculation resulted in developing a subcutaneous solid tumor of a size ranging in diameter from 0.3 to 0.5 cm 6 days later. The mice were divided into several groups, composed of 8 animals, some of which died during the experimental procedures and were excluded from the results. On the 6th day after inoculation the experimental groups were subjected to hypothermia of 20°C for 6 or 10 hours and then administered with one of the following anticancer agents. The anticancer drugs used were Endoxan and Mitomycin C. The total dose of the drugs per mouse was 80 mg/kg of Endoxan or 3 mg/kg of Mitomycin C, each roughly corresponding to a half of LD 50 of the drugs. These drugs were dissolved in phisiological saline solution, so that each 0.1 cc might contain one third of the total dose of the drugs, which was injected intraperitoneally into each mouse three times every hour.

The growth of tumors and survival days of animals in the experimental groups were compared with those in the four control groups ; 1) without any treatment, 2) subjected merely to hypothermia, 3) treated merely with the anticancer drugs, and 4) injected with the drugs during hypothermia.

Classification of the groups was as follows.

A) Groups of Sarcoma 180 bearing mice

1. Control-group I (without any treatment)

The mice were treated neither with hypothermia nor with the anticancer drugs.

2. Control-group 11 (treated with 6 hours of hypothermia alone)

The mice were subjected to 6 hours of hypothermia at 20°C without administration of the anticancer drugs.

3. Control-group III (injected with the anticancer drug without hypothermia)

a) injected with Mitomycin C.

Each mouse received a total of 3 mg/kg of Mitomycin C which was divided into 3 doses, each being given at hourly intervals.

b) injected with Endoxan.

Each mouse received a total of 80 mg/kg of Endoxan given in the same way as in the group injected with Mitomycin C.

4. Control-group IV (injected with Endoxan during 6 hours of hypothermia)

A total of 80 mg/kg of Endoxan was divided in 3 doses, each being given at hourly intervals, starting immediately after the beginning of hypothermia.

5. Experimental-group I (injected with Mitomycin C 2 hours after 6 hours of hypothermia)

A total of 3 mg/kg of Mitomycin C was divided into 3 does, each being given at hourly intervals, starting 2 hours after rewarming.

6. Experimental-group II (injected with Mitomycin C hours after 6 hours of hypothermia)

The same dose of Mitomycin C was administered in the same way as in Experimental Group I, starting 5 hours after rewarming.

7. Experimental-group III (injected with Endoxan 2 hours after 6 hours of hypothermia)

A total of 80 mg/kg of Endoxan was administered in the same way as in the case of Mitomycin C, starting 2 hours after rewarming.

8. Experimental-group IV (injected with Endoxan 5 hours after 6 hours of hypothermia)

The same dose of Endoxan as in Experimental-group III was administered similarly, starting 5 hours after rewarming.

9. Experimental-group V (ingected with Endoxan after 10 hours of hypothermia)

The same dose of Endoxan as in Experimental-group III was administered similarly, starting 6 hours after rewarming.

B) Groups of Ehrlich carcinoma bearing mice

1. Control-group I (without any treatment)

2. Control-group II (treated with 6 hours of hypothermia alone)

3. Control-group III (injected with Endoxan without hypothermia)

A total of 80 mg/kg of Endoxan was divided into 3 doses, each being given at hourly intervals.

4. Control-group IV (injected with Endoxan during 6 hours of hypothermia)

The same dose of Endoxan as in Control-group III was administered similarly, starting immediately after the beginning of hypothermia.

5. Experimental-group I (injected with Endoxan 2 hours after 6 hours of hypothermia)

A total of 80 mg/kg of Endoxan was administered in the same way as in Control-group III, starting 2 hours after rewarming.

6. Experimental-group II (injected with Endoxan 5 hours after 6 hours of hypothermia)

The same dose of the drug as in Control-grup III was administered similarly, starting 5 hours after rewarming.

In each group, the growth of tumors was recorded by measuring two diameters of the tumors at intervals of 3 to 5 days after the treatments, and the survival time of the animals was also recorded.

2. RESULTS

A) Groups of Sarcoma 180 bearing mice

1) Control-group I & II

In Control-group I which received no treatment, the tumors showed a steady increase in size, finally killing all the mice 21 to 60 days after tumor inoculation (Fig. 23). In Control-group II which was subjected to 6 hours of hypothermia, the rate of tumor growth seemed to be suppresed at first. After that time, however, the tumors showed a rapid increase in size and finally killed the animals 21 to 59 days after inoculation (Fig. 24).

2) Control-group III (a)

Although tumor growth was more markedly suppresed than in Control groups I & II, the tumors gradually increased in size until they killed their hosts 44 to 64 days after inoculation (Fig. 25).

3) Control-group III (b)

The tumor-inhibiting effect of Endoxan on Sarcoma 180 is presented in Fig. 26. Although tumor growth was more markedly inhibited in this group than in Control-group III (a) treated with Mitomycin C, the survival time of the animals was almost the same, ranging from 24 to 62 days after inoculation.

4) Control-group IV

In this group the mice showed a more remarkable decrease in the rate of tumor growth after the treatment, but their survival times, ranging from 23 to 63 days

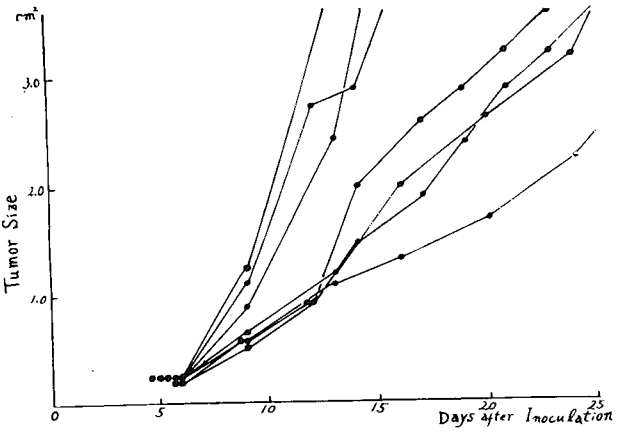

Fig. 23 Growth curve of Sarcoma 180 solid tumor in Control-group I without any treatment.

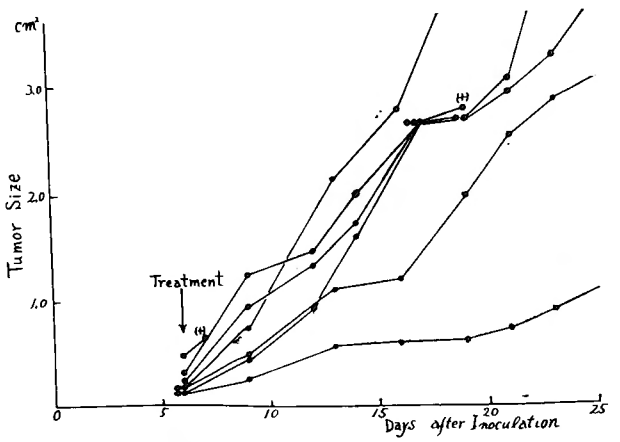

Fig. 24 Growth curve of Sarcoma 180 solid tumor in Control-group II treated with 6 hours hypothermia alone.

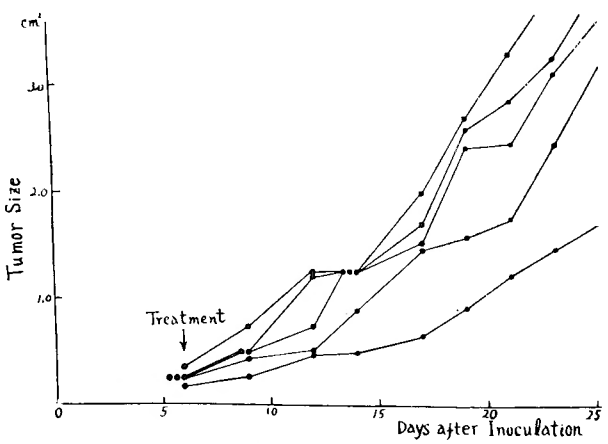

Fig. 25 Growth curve of Sarcoma 180 solid tumor in Control-group III(a) treated with 3 mg/kg of Mitomycin C alone.

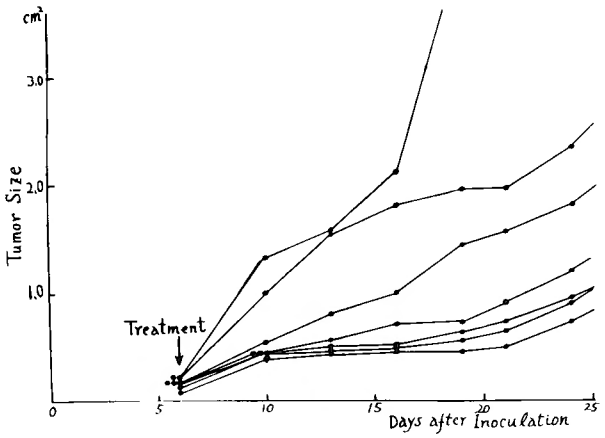

Fig. 26 Growth curve of Sarcoma 180 solid tumor in Control-grouq III(b) treated with 80 mg/kg of Endoxan alone.

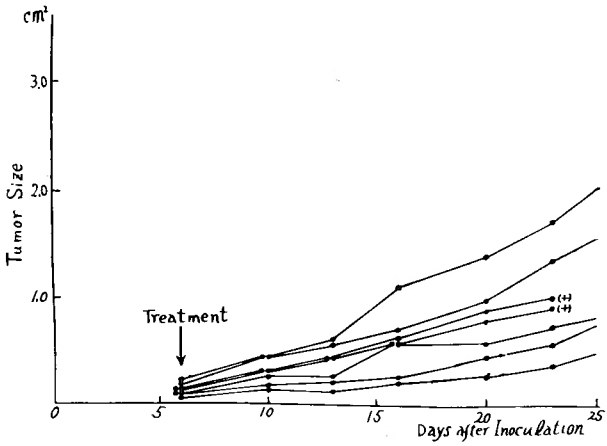

Fig. 27 Growth curve of Sarcoma 180 solid tumor in Control-group IV treated with 80 mg/kg of Endoxan during 6 hours hypothermia.

after inoculation, were almost the same as those observed in Control-group III (b) (Fig. 27).

5) Experimental-groups I & II

The inhibition of tumor growth in both groups was much more striking than in Control group III (a) treated with Mitomycin C alone (Figs 28 & 29). In particular, one of 6 mice in Group I and 2 of 7 mice in Group II showed complete regression of tumor 20 days after the treatment, and any sign of reccurrence was not observed for 3 months thereafter. There was no significant difference in the rate of tumor growth between the two experimental groups.

Comparing the survival times in these groups with those in Control-group III (a), it was found that the mice in the experimental groups survived longer than those in Control-group III (a) (Fig. 30).

6) Experimental-groups III & IV

In both of the groups, in which the injection of Endoxan was begun 2 or 5 hours after rewarming, tumor growth was inhibited much more markedly than in the control groups (Figs. 31 & 32). Particularly in Experimental-group IV, in which the injection was begun 2 hours after rewarming, inhibition of tumor growth was observed to be the strongest, resulting in a complete cure in 2 of 7 mice.

7) Experimental-group V

In this group, it was observed that tumor growth was, in every mouse, suprressed most markedly, and 3 of the 8 tumors soon regressed and completely disappeared in about 20 days after the treatment (Fig. 33).

In Fig. 34 are presented the results of comparison between the survival days of mice in this group and those in Control-group III (b) treated with Endoxan alone and Experimental-groups III & IV given the drugs after 6 hours of hypothermia. The mice in this

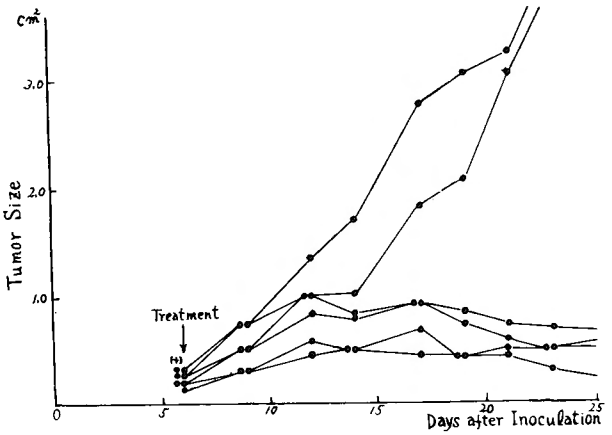

Fig. 28 Growth curve of Sarcoma 180 solid tumor in Experimental-group I treated with 3 mg/kg of Mitomycin C 2 hours after release from 6 hours hypothermia.

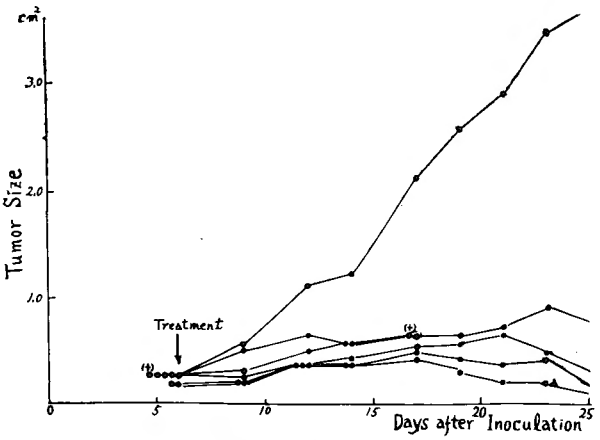

Fig. 29 Growth curve of Sarcoma 180 solid tumor in Experimental-group II treated with 3 mg/kg of Mitomycin C 5 hours after release from 6 hours hypothermia.

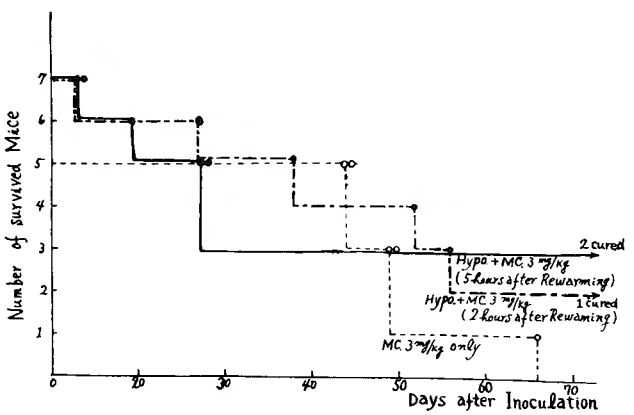

Fig. 30 Survival days of Sarcoma 180 bearing mice treated with Mitomycin C.
·····: Control-group III (a) treated with Mitomycin C alone.
●- -●: Exp.-group I treated with Mitomycin C 2 hours after 6 hours hypothermia.
●——●: Exp.-group II treated with Mitomycin C 5 hours after 6 hours hypothermia.

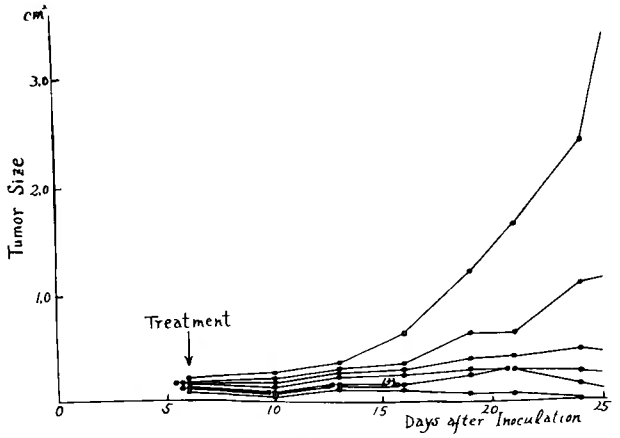

Fig. 31 Growth curve of Sarcoma 180 solid tumor in Experimental-group III treated with 80 mg/kg of Endoxan 2 hours after release from 6 hours hypothermia.

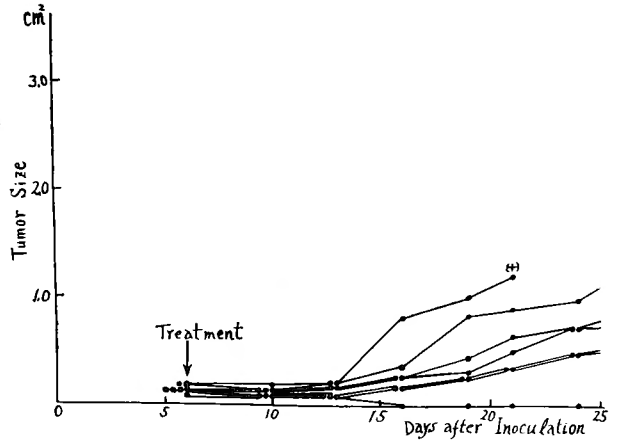

Fig. 32 Growth curve of Sarcoma 180 solid tumor in Experimental-group IV treated with 80 mg/kg of Endoxan 5 hours after release from 6 hours hypothermia.

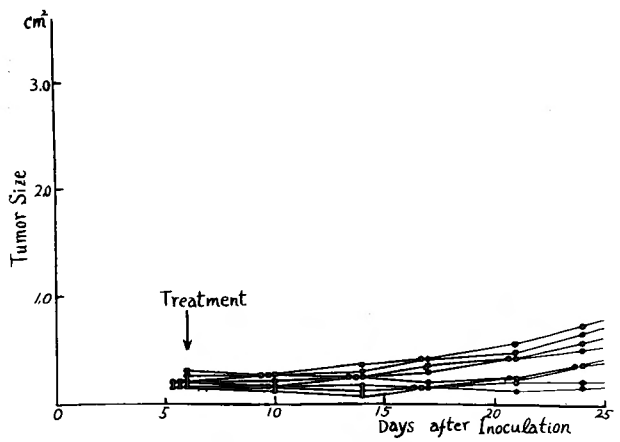

Fig. 33 Growth curve of Sarcoma 180 solid tumor in Experimental-group V treated with 80 mg/kg of Endoxan 6 hours after release from 10 hours hypothermia.

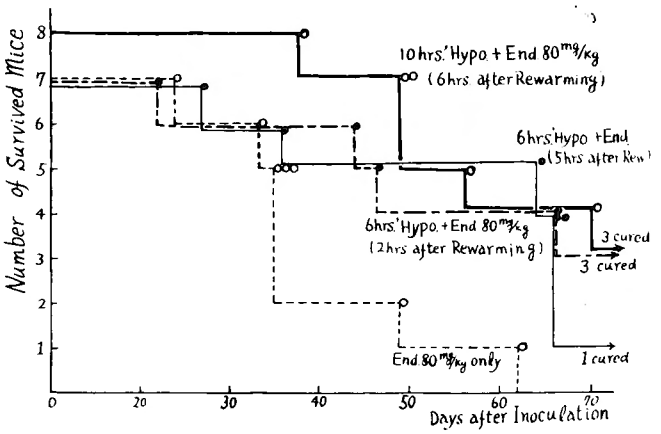

Fig. 34 Survival days of Sarcoma 180 bearing mice treated with Endoxan.
○·····○ : Control-group III (b) treated with Endoxan alone.
● · ● : Exp.-group III tseated with Endoxan 2 hours after 6 hours hypothermia.
●—·—● : Exp.-group IV treated with Endoxan 5 hours after 6 hours hypothermia.
—— : Exp.-group V treated with Endoxan 6 hours after 10 hours hypothermia.

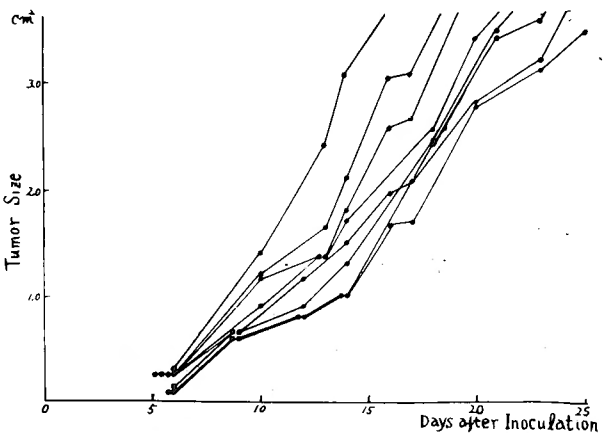

Fig. 35 Growth curve of Ehrlich solid carcinoma in Control-group I without any treatment.

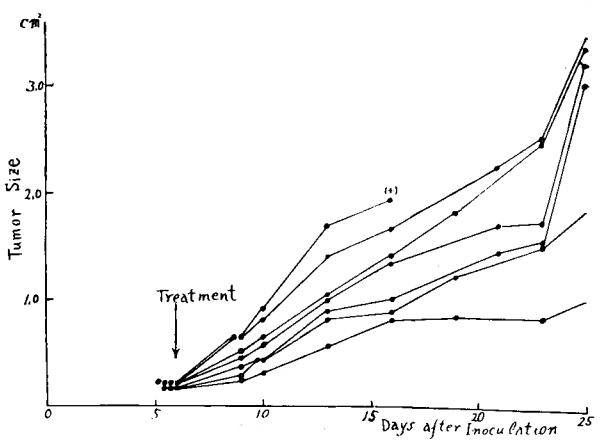

Fig. 36 Growth curve of Ehrlich solid carcinoma in Control-group II treated with 6 hours hyp... thermia alone.

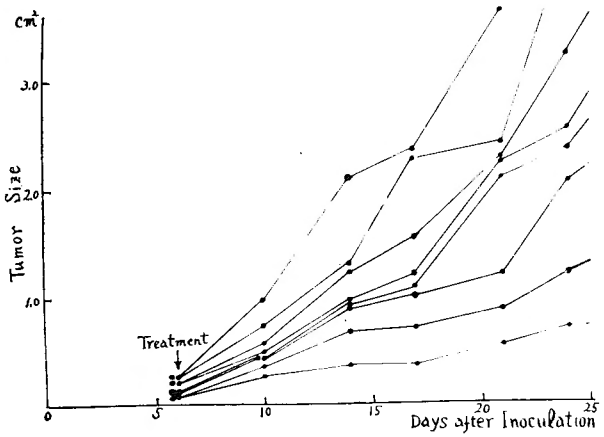

Fig. 37 Growth curve of Ehrlich solid carcinoma in Control-group III treated with 80 mg/kg of Endoxan alone.

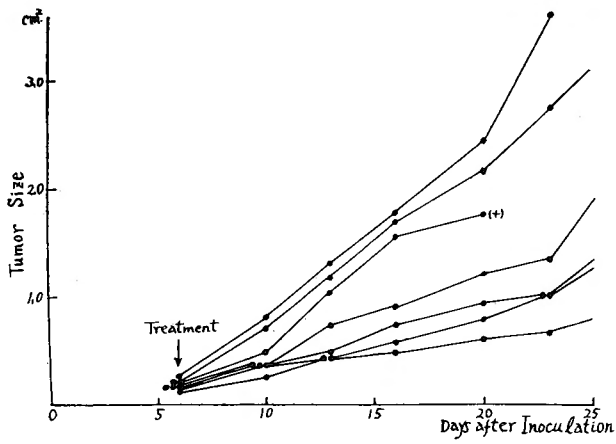

Fig. 38 Growth curve of Ehrlich solid carcinoma in Control-group IV treated with 80 mg/kg of Endoxan during 6 hours hypothermia.

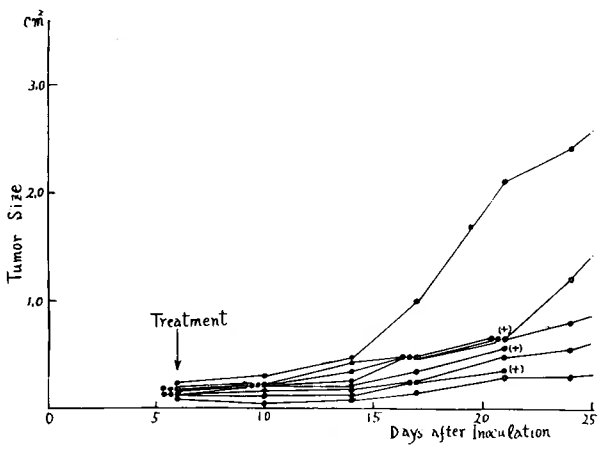

Fig. 39 Growth curve of Ehrlich solid carcinoma in Experimental-group I treated with 80 mg/kg of Endoxan 2 hours after release from 6 hours hypothermia.

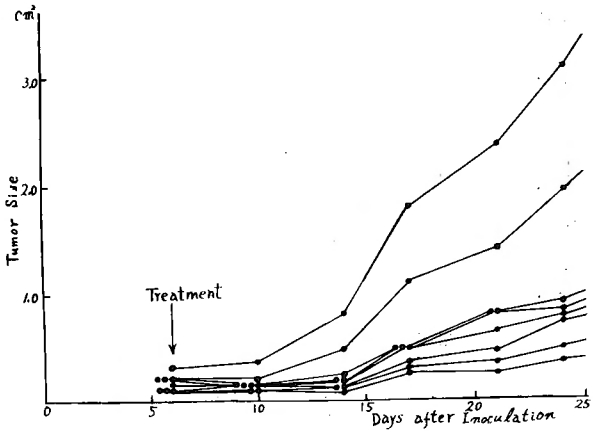

Fig. 40 Growth curve of Ehrlich solid carcinoma in Experimental-group II treated with 80 mg/kg of Endoxan 5 hours after release from 6 hours hypothermia.

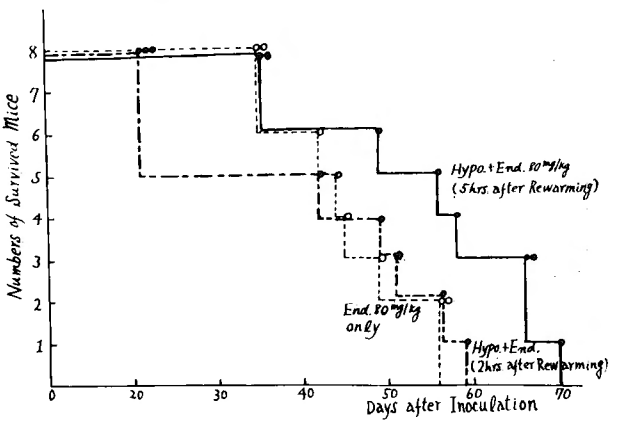

Fig. 41 Survival days of Ehrlich carcinoma bearing mice treated with Endoxan.
○·····○ : Control-group III treated with Endoxan alone.
●-·-·-● : Exp.-group I treated with Endoxan 2 hours after 6 hours hypothermia.
●——● : Exp.-group II treated with Endoxan 5 hours after 6 hours hypothermia.

group survived far longer than those in the other groups, showing a complete regression of tumors in 3 of the 8 animals. As for Experimental-groups III & IV which were given the drugs after 6 hours of hypothermia, most of the animals showed a remarkable prolongation in their survival days, as compared with those in Control-group III (b) given the drug alone.

B) Groups of Ehrlich carcinoma bearing mice

1) Control-groups I & II

In Control-group I which received no treatment, the tumors were obsevred to grow somewhat more rapidly than in the case of Sarcoma 180, causing all the mice to die 25 to 41 days after inoculation (Fig. 35). In Control-group II which was treated with hypothermia alone, tumor growth was at first suppressed to some degree, until a sudden increase in the size of the tumors was observed about 20 days after inoculation (Fig. 36).

There was no significant difference between the survival times of Control group I and that of Control-group II, each ranging from 23 to 45 days.

2) Control-groups III & IV

In Control-group III, Endoxan in a total dose of 80 mg/kg caused a moderate inhibition of tumor growth, though with a great disparity in its effect, allowing the animals to live for the period of 35 to 56 days after inoculation (Fig. 37).

In Control-group IV which was given the drug during hypothermia, inhibition of tumor growth was observed to be a little stronger than in Control-group III. However, the survival times of the mice in Control-group IV, each ranging from 35 to 62 days, were almost similar to those observed in Control-group III (Fig. 38).

3) Experimental-groups I & II

In these groups, the tumors were observed to grow very slowly for the first 20 days after inoculation, as compared with those in the control groups. Thereafter, however, they showed a steady increase in size, and finally killed all the mice (Figs. 39, 40).

Survival days in these groups are compared with those in Control-group III (Fig. 41). It is noticed that most of the mice in Experimental-group II given the drug 5 hours after rewarming survived somewhat longer than those in Control-group III.

3. DISCUSSION

Since long ago a great interest has been taken by many investigators in a conjecture that such young undifferentiated cells as tumor cells would require an optimal temperature for their growth and proliferation, and that these cells would be more readily affected by alternation in temperature than differentiated cells of adult type. Malignant tumors have been reported to be rarely accompanied with feverrish diseases such as malaria, erysipelas etc[2]. On the other hand, COLEY[8], MASON[23] etc, found that both primary and metastatic carcinoma were extremely uncommon in the extremities where the body temperature was fairly lower than in other portions of the body. These reports might suggest that growth of tumor cells is readily influenced by abnormal temperatures. Taking this point of view into consideration, a number of investigators have tried to control tumor growth by means of temperature shifts per se. In the present experiment hypothermia has been utilized to induce in-vivo synchronization of tumor cells.

According to NEWTON et al[28]. synthesis of DNA in HeLa cells exposed to a low temperature of 4°C increased immediately before synchronous cell division. And it was indicated that synchrony in cell division is preceded by synchrony in DNA synthesis.

Many studies have so far been made on the period of DNA synthesis in the division cycle, which seems to vary with the kind of cells. In some bacteria such as E. Coli and Salmonella typhimurium, SCHAECHTER[30] and MC-FALL[26] found that DNA was synthesized continuously or almost continuously throughout the entire division cycle. In most animal and plant cells, however, as reported by SWIFT[39] and VENDRELY[42], the duplication of DNA takes place in late interphase, while in some blastula cells[12] it was reported to occur in the beginning of interphase. As for tumor cells, it was demonstrated by EDWARDS[10] and HOWARD[16] that DNA synthesis in Ehrlich ascites cells was performed mainly in late interphase, beginning several hours after the completion of mitosis and ending just before

the following mitosis. In synchronously dividing HeLa cells, SMITH et al[36]. observed that DNA synthesis occured in two periods in interphase; in the first 4 hours after mitosis DNA increased by 40% and the remaining 60% increase occured 3 to 5 hours before the following mitosis.

In Experiment I, a marked peak in the curve of mitotic index was found 4, 8 and 12 hours after rewarming, respectively in the cases of 2, 6 and 10 hours of hypothermia. These results led us to have a presumption that a considerable amount of DNA was synthesized during a short period of time immediately before the appearence of the first peak. This presumption agrees with the observations by TAKAHASHI[49] that when tumor-bearing mice were subjected to 6 hours of hypothermia at 20°C, the P^{32} incorporation into DNA in the tumor tissue was maximum in the period between 2 and 4 hours after rewarming.

The mechanism of the anticancer effect of Mitomycin C was reported to inhibit selectively the biosynthesis of DNA in the resting nuclei of tumor cells.[4][19] On the other hand, Endoxan is one of cyclic phosphamide esters of nitrogen mustard which is designed to have an inactive "tranport form" in order to increase its selective affinity for tumor cells. The main effect of these derivatives of nitrogen mustard on tumor cells is also thought to inhibit selectively DNA synthesis without exerting any damage on RNA synthesis[1][3][35]. It would be reasonable to expect that these drugs, when administered to the artificially synchronized tumor cells in the period of their synchronized synthesis of DNA, would result in greater therapeutic effects. Endoxan or Mitomycin C was administered to the tumor-bearing mice three times every hour, starting 2 or 5 hours after the release from 6 hours of hypothermia. A group of Sarcoma 180 bearing mice which were subjected to 10 hours of hypothermia was also treated with Endoxan in the same way 6 hours after rewarming. The results were compared with those in the control groups.

Firstly, comparisons were made between the results in the non-treated groups and those in the cooled groups without the administration of the drugs. It was revealed that in the latter case, although tumor growth in Sarcoma 180 as well as in Ehrlich carcinoma was at first supressed to some degree, the tumors increased rapidly in size, finally killing all the mice in the same period of time after inoculation as in the former case. These observations agree with the findings observed in Experiment I that hypothermia of this degree caused no irreversible degenerative changes in the tumor cells.

Secondly, when the results in the groups given the drugs after hypothermia were compared with those in the groups given the drugs alone or those in the groups given the drugs during hypothermia, it was indicated that the former groups showed much more remarkable inhibition of tumor growth than the latter two groups. Particularly in the groups which were bearing Sarcoma 180, there was a marked prolongation in the survival days of mice, accompanied with complete regression of the tumors in some cases. In case of Ehrlich carcinoma, however, the effect of the treatment was confined within a short period of time after treatment and the survival days of the animals showed little significant difference from those in the control groups. This is probably due to the low degree of synchrony of mitosis in the tumor cells, in consequence of which the drugs are surmised not to have been able to affect so many tumor cells.

Between each of the two groups which were treated with the drugs 2 or 5 hours

after 6 hours of hypothermia, there was no difference worth noticing in the tumor inhibiting effect. Further examination is required to determine an appropriate time for administration of anticancer drugs to acquire more satisfactory therapeutic benefits.

Thirdly, in the group which received Endoxan after 10 hours of hypothermia, tumor growth was more strongly inhibited and the survival days of the mice were more markedly prolonged than in the group treated in the same way after 6 hours of hypothermia. These findings would indicate that a better synchrony of mitosis in tumor cells can result in a greater therapeutic benefit.

4. SUMMARY

1) Mice which were bearing Sarcoma 180 solid tumor or Ehrlich solid carcinoma inoculated subcutaneously 6 days previously were subjected to hypothermia at 20°C for 6 hours. Then the mice were returned to normal body temperature. Two or 5 hours after the rewarming, they were given a total dose of 3 mg/kg of Mitomycin C or of 80 mg/kg of Endoxan which was divided into three doses, each being injected intraperitoneally at hourly intervals. Another group of mice which were bearing Sarcoma 180 of subcutaneous solid form were subjected to 10 hours of hypothermia at the same temperature, and then treated with 80 mg/kg of Endoxan given in the same way, starting 6 hours after rewarming. Tumor growth as well as survival days of the mice observed in these experimental groups were compared with those in the following four control groups; the first group without any treatment, the second group treated with 6 hours hypothermia alone, the third group treated with the drugs alone, and the fourth group treated with the drugs during hypothermia.

2) In the experimental groups injected with the drugs after 6 hours of hypothermia, there was a marked inhibition of tumor growth with prolonged survival days of the mice, as compared with the control groups.

3) In the experimental group which was subjected to 10 hours of hypothermia, Endoxan administered after rewarming caused a greater inhibitory effect on the growth of Sarcoma 180 and prolonged the survival time of the animals more markedly than in the case of 6 hours of hypothermia.

4) From these findings, it can be said that the longer the duration of hypothermia was, the greater was the degree of synchrony in mitosis, which resulted in greater therapeutic effects of anticancer drugs.

CONCLUSION

Mice of dd strain which were bearing Sarcoma 180 or Ehrlich carcinoma were subjected to hypothermia at 20°C for varying periods of time, and then returned to normal body temperature. Histological examination on the changes in the mitotic index in tumor tissue revealed that a certain degree of in-vivo synchronization of mitosis was induced in the tumor cells and that hypothermia of a longer duration could result in a better synchrony. Anticancer agents, Mitomycin C or Endoxan, which are supposed to inhibit chiefly DNA synthesis, were administered to the tumor-bearing mice shortly after the hypothermia.

It was proved that the anticancer effect of the drugs could be intensified when the drugs were given to the synchronized tumor cells in the late interphase in which a considerable amount of DNA was supposed to be synthesized.

I would like to express my deep gratitude to Assist Prof. Dr. Ikuzo Yokoyama for his cordial guidance throughout this experiment.

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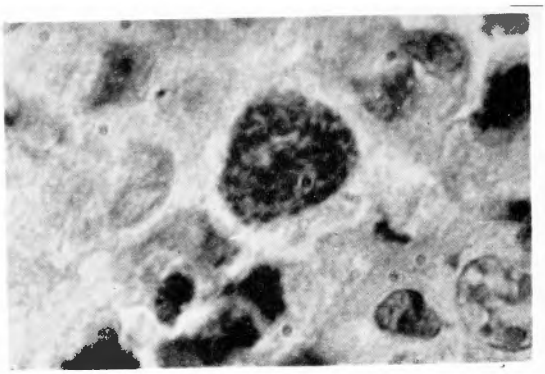

Fig. 2 Sarcoma 180 cell in late prophase. ×1500

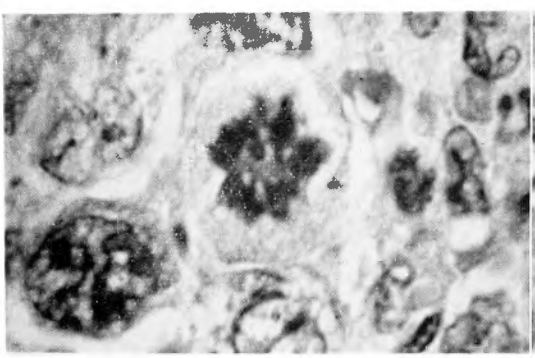

Fig. 3 Sarcoma 180 cell in metaphase seen from the direction of the pole. ×1500

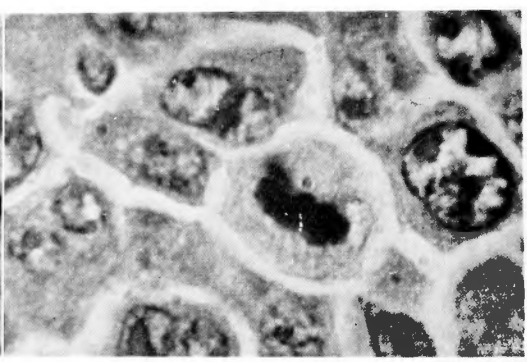

Fig. 4 Sarcoma 180 cell in metaphase seen from the direction of the equator. ×1500

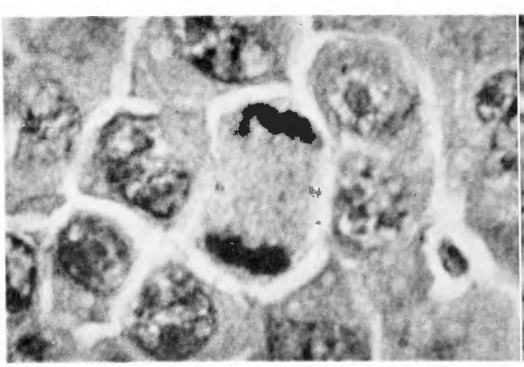

Fig. 5 Sarcoma 180 cell in anaphase. ×1500

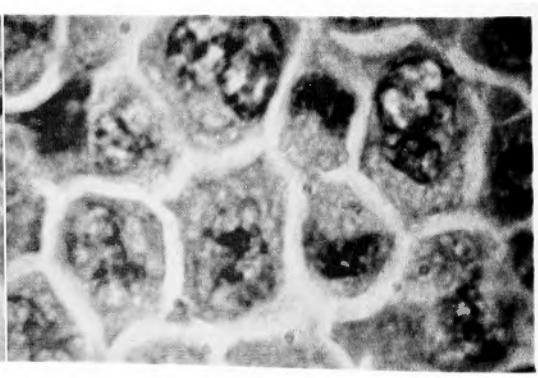

Fig. 6 Sarcoma 180 cell in telophase. ×1500

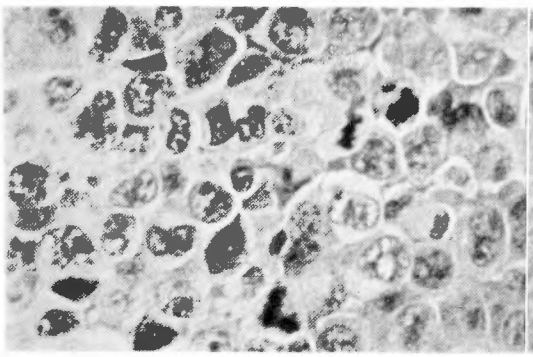

Fig. 7 Sarcoma 180 solid tumor, before cooling. ×600.

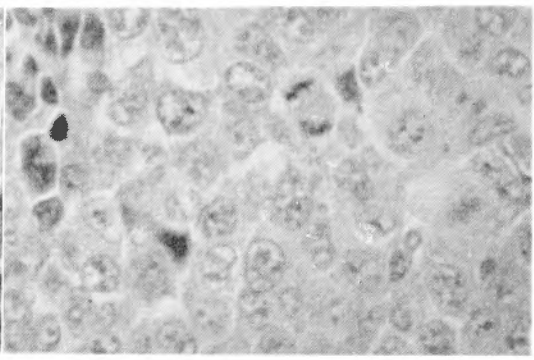

Fig. 8 Sarcoma 180 solid tumor, immediately after 2 hours hypothermia at 20°±2°C. No degenerative changes in tumor cells, still accompanied with many mitotic figures. ×600.

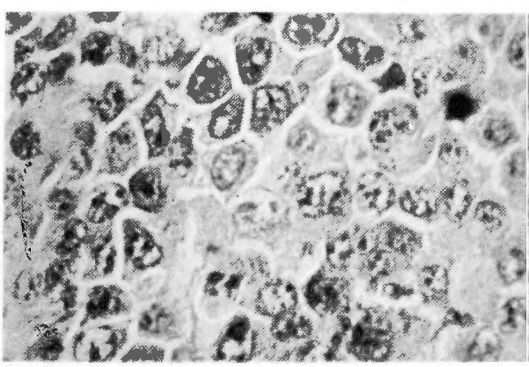

Fig. 9 Sarcoma 180 solid tumor, immediately after 6 hours hypothermia at 20°±2°C. Little degenerative changes in tumor cells. ×600.

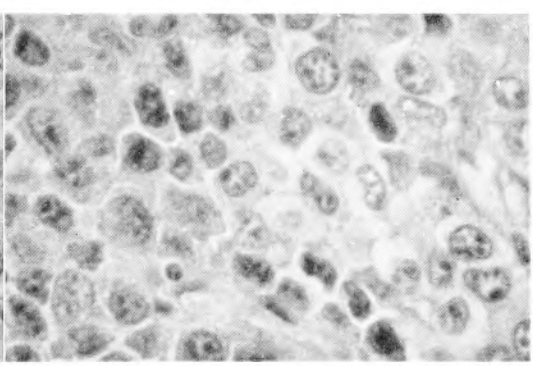

Fig. 10 Sarcoma 180 solid tumor, immediately after 10 hours hypothermia at 20°±2°C.
Slight degenerative changes such as condensation of nuclei and vacuolar formation of cytoplasm, with mitotic figures in markedly decreased number. ×600.

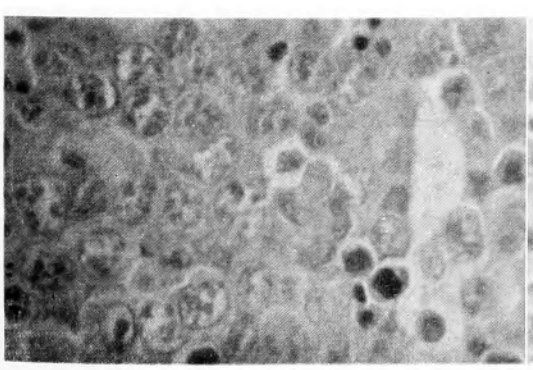

Fig. 11 Sarcoma 180 solid tumor, 4 hours after release from 2 hours hypothermia at 20°±2°C. ×600.

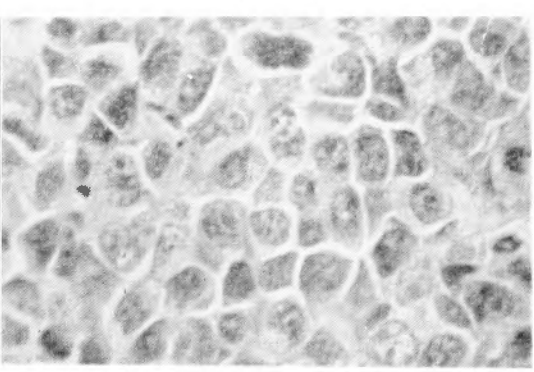

Fig. 12 Sarcoma 180 solid tumor, 8 hours after release from 6 hours hypothermia at 20°±2°C.
Almost same findings as before cooling. ×600.

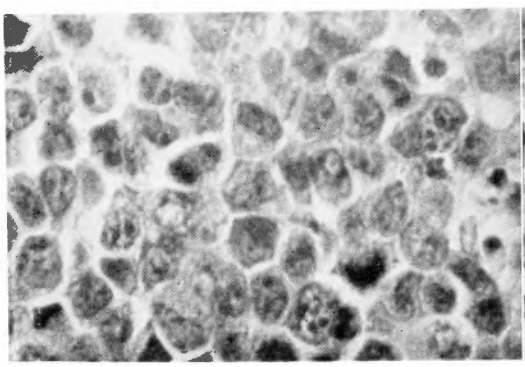

Fig. 13 Sarcoma 180 solid tumor, 12 hours after release from 10 hours hypothermia at 20°±2 C
Mitotic figures in larger number than before or during hypothermia. ×600.

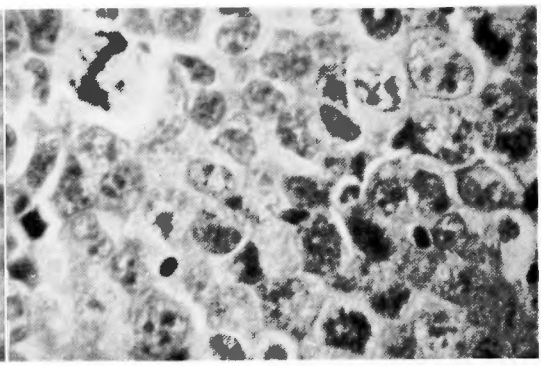

Fig. 14 Sarcoma 180 solid tumor, 24 hours after release from 10 hours hypothermia at 20°±2 C.
Almost none of such degenerative changes in tumor cell as during hypothermia. ×600.

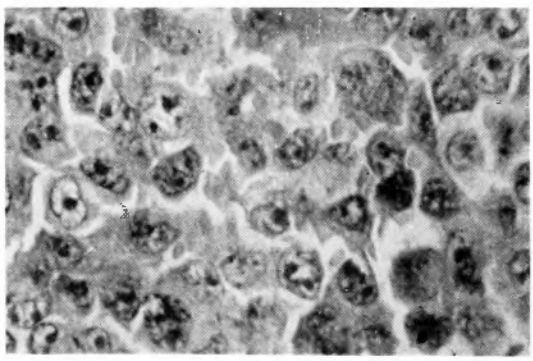

Fig. 18 Ehrlich solid carcinoma. Before cooling. ×600.

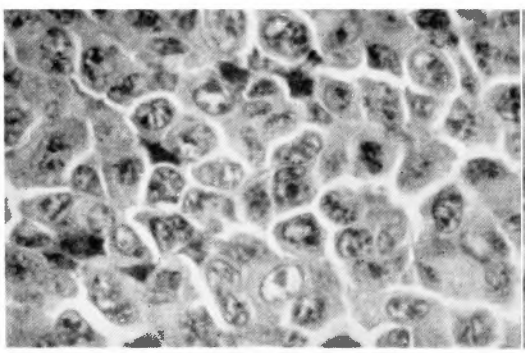

Fig. 19 Ehrlich solid carcinoma, immediately after 6 hours hypothermia at 20°±2 C. Little degenerative changes in tumor cells. ×600.

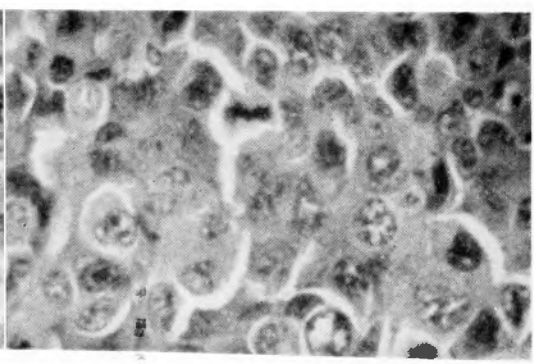

Fig. 20 Ehrlich solid carcinoma, 8 hours after release from 6 hours hypothermia at 20°±2 C.
Almost same picture as before cooling, expcepting a marked increase in number of mitotic cells. ×600.

和文抄録

低体温法の制癌化学療法への応用に関する実験的研究

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加藤忠雄

1955年，Seherbaum 及び Zeuthen が Tetrahymena pyriformis について，その環境温度を変えると一定時間後に大多数の細胞に同調的分裂を生ぜしめ得る事を発見して以来，温度刺戟による細胞の人為的同調分裂に関する実験は，細菌類より動植物細胞に到る迄数多く試みられている．しかしこれらは何れも in vitro の実験であり，in vitro の細胞分裂同調化に関する研究は殆んど報告されていない．著者は固形型の実験腫瘍を用い，腫瘍細胞の分裂を生体内で人為的に同調させ，且つこれを癌化学療法に応用しようと試みた．即ち担癌マウスに一定の低体温を実施し，冷却中並びに復温後に於ける腫瘍細胞の有糸分裂指数の変動を追求する事により，in vivo に於ける腫瘍細胞の同調的分裂の有無を検索すると共に，低体温の持続時間を種々に変えて同調化の増強をはかつた．更に，これら担癌マウスに復温後の一定期に制癌剤を投与して，その治療効果の増強の程度を比較検討した．

先ず，Sarcoma 180 腹水腫瘍細胞皮下移植後10日目のマウスに，Nembutal 37.5mg/kg，及び Wintermin 15mg/kg を投与して，20℃ の低体温を夫々2時間，6時間，10時間施行し，ついで 38℃ の常体温に復帰せしめた．この間，経時的に腫瘍組織標本を採取して，Haematoxylin-Eosin 重染色を施し，その組織学的所見殊に有糸分裂指数の変遷を観察した．又，Ehrlich 腹水癌細胞皮下移植マウスに同温度の低体温を6時間施行して，同様な観察を行なつた．

1) 両固形皮下腫瘍とも，低体温開始と同時に徐々に分裂指数の減少を示すが，低体温3時間にしてその値は急減し，以降10時間迄冷却を続けても分裂指数は略一定値を保持して著明な変動を示さなかつた．この間，分裂中期の細胞が比較的多くみとめられ，復温後は分裂後期以降の細胞の増加が割合著明であつた．これは低体温により，分裂に入らんとする細胞が抑制されると共に分裂中の細胞，特に分裂中期にある細胞の分裂時間が延長される為で，低体温は分裂直前の細胞に最も強く作用するが分裂中の細胞に関しては中期より後期への移行期に強く作用するものと考えられる．

2) 復温後の経過をみると，2時間低体温群では復温後4時間目に，6時間低体温群では8時間目に，又10時間低温群では12時間目に，それぞれ，分裂指数の最高値（peak）が現われ，低体温の時間を延長すれば，その時間だけこの peak の出現が遅れ，且つ peak の上昇度が著明になつた．即ち，低体温の時間の長いもの程，より多数の腫瘍細胞が同調的に分裂するものと考えられる．

3) 分裂指数の最初の peak が出現してから，当該腫瘍細胞の分裂週期と略々等しい期間，約20時間後に第2の peak が出現し，その値は最初の peak のそれと略々同値であつた．

4) 以上の実験成績より，in vivo に於いても in vitro 同様，Zeuthen et al. らの同調的細胞分裂と同様な状態を実験的に誘起させる事が出来，且つこの同調化は少く共2つの細胞周期にわたり続き，更に低体温の持続時間を延長すればそれだけ同調化はより完全となる事が判明した．

5) 低体温によつて生じた軽度の細胞変性は復温後時間の経過と共に漸次消褪し，この範囲の低温刺戟では腫瘍細胞に著明な不可逆的変性を来すものではない事が判明した．

かくの如く，低体温により，生体内の腫瘍細胞に或る程度の同調的分裂を誘起する事が出来たが，この際同時に DNA 合成も或る程度同調化し，且つその合成は同調的細胞分裂の直前にて比較的短時間内に起きるものと考え，この時期を狙つて DNA 合成阻害が主作用と考えられる Mitomycin C，又は Endoxan を集中投与して，その制癌効果を高めんとした．即ち，これら担癌マウスに移植後6日目，20℃，6時間の低体温を実施し，復温後2時間目，又は5時間目より，

Mitomycin C 3 mg/kg，若くは Endoxan 80mg/kg を1時間間隔で3回に分割投与して，以後の腫細の大きさ及び担癌マウスの延命日数に及ぼす効果を観察した．又 Sarcoma 180群の一部には，20°C，10時間の低体温を施行して，復温後6時間目より Endoxan 80mg/kg を同様に3回に分注してその効果を観察した．同時に対照群として，無処置群，低体温単独施行群，薬剤単独投与群，及び低体温中薬剤投与群を作り，その成績を比較観察した．

1) 低体温後薬剤投与群では対照群に比べて，腫瘍発育抑制効果及び延命効果の増強がみとめられた．特に Sarcoma 180群では，Mitomycin C, Endoxan 投与群とも少数ながら腫瘍の完全な退縮をもたらしたものがあつた．

2) 10時間低体温併用群では，6時間低体温群に比べて腫瘍発育抑制効果及び延命効果において，更に強い増強がみとめられ，腫瘍細胞分裂の同調化の程度をたかめると，それに伴なつて治療効果も増強する事が判明した．