In-Vivo Synchronous Mitosis of Cancer Cells Induced by Hypothermia and its Application to Cancer Chemotherapy

by

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INTRODUCTION

Since the pioneering studies of SCHERBAUM and ZEUTEN, numerous examples of in-vitro synchronous cell division induced artificially by temperature shift have been reported [3)7)26)36)]. Most studies in this field were carried out on microorganisms, protozoa or animal eggs in early cleavage stages. As to cells of mammalian origin, NEWTON and WILDY attempted to induce synchronization of mitosis in cultures of HELA cells[26)]. All these investigations have been performed in vitro. As for in-vivo synchronous mitosis of mammalian cells, little work has been done and to our knowledge, nobody has ever tried to utilize this kind of phenomenon in the field of cancer treatment. TAKAHASHI showed that when mice which were bearing NF sarcoma were subjected to a hypothermia of 20°C for six hours, DNA synthesis in the tumor tissue was the most vigorous a few hours after the hypothermia[42)]. These observations led us to presume that after being subjected to hypothermia, cancer cells might also be synchronized in vivo in regard to mitosis.

Each cancer cell is in its individual phase of the division cycle and divides at random. Radiomimetic alkylating agents are thought to be the most effective to cancer cells in their premitotic stage[4)]. When mitosis of cancer cells can be synchronized in vivo by some means such as hypothermia and the anticancer agents are administered to the synchronized cells in the most sensitive phase, the effect of the agents can be intensified.

This study has been carried out to examine whether our work hypothesis mentioned above is applicable in an experimental chemotherapy of mouse tumor. Mice which were bearing an ascitic tumor were subjected to hypothermia, and changes in mitotic index in the ascites were examined during and after the hypothermia. At the same time, a new method to intensify the effect of alkylating agent by taking advantage of the phenomenon of in-vivo synchronous mitosis was applied to an experimental chemotherapy on the tumor-bearing mice.

PART I

MITOTIC COUNTS IN EHRLICH ASCITES CANCER DURING AND AFTER HYPOTHERMIA

MATERIALS AND METHODS

Male mice of dd strain, about fifty days old and each weighing 20 to 25 grams

were used. They were given a standard compressed diet and drinking water ad libitum. Ehrlich 10-day-old ascites cancer cells were transplanted intraperitoneally to the mice. The rate of transplantability of the tumor was almost 100%, when one million cells were inoculated.

Four days after the intraperitoneal inoculation of 15 million tumor cells, the mice were injected intramuscularly with 50mg of Nembutal per kilogram of body weight. The mice were divided into three groups, each composed of 3 animals, according to the degree of hypothermia which was to be given to the animals. Twenty minutes after the administration of Nembutal, the mice were placed on a tray in an ice box. Their body temperature was measured with a electric thermometer, the thermocouple being inserted in the rectum 1.5cm deep from the anus.

In the first group (Fig. 1), the body temperature was lowered so that it might reach 20°C 30 minutes after the beginning of hypothermia. When the body temperature was reduced to 20°C, the mice were removed and placed on another tray which was kept at the usual room temperature. When necessary, the mice were brought back to the cold tray. In this way, the low temperature was maintained at the same level of 20°C for six hours. In the second group (Fig. 2), hypothermia was repeated. Mice which had already been subjected to a hypothermia of 20°C for six hours as in the first group, were rechilled 12 hours after being released from the first hypothermia, the rectal temperature

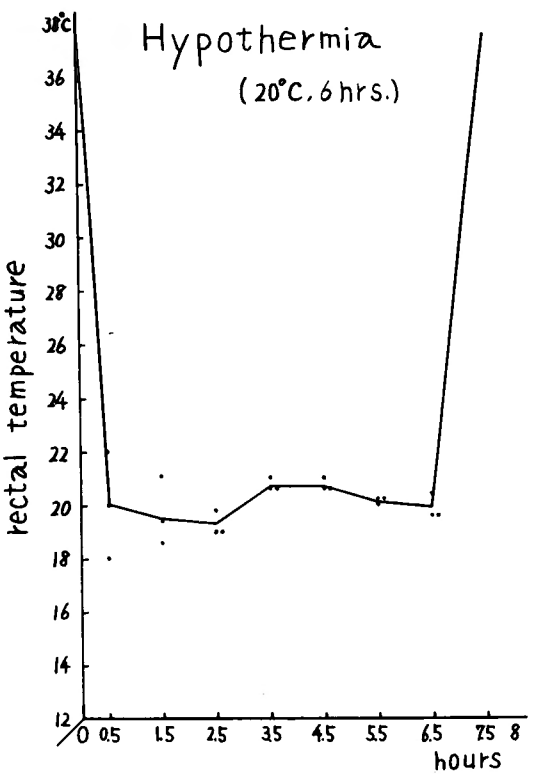

Fig. 1 Rectal temperature of mice subjected to hypothermia at 20°C for six hours

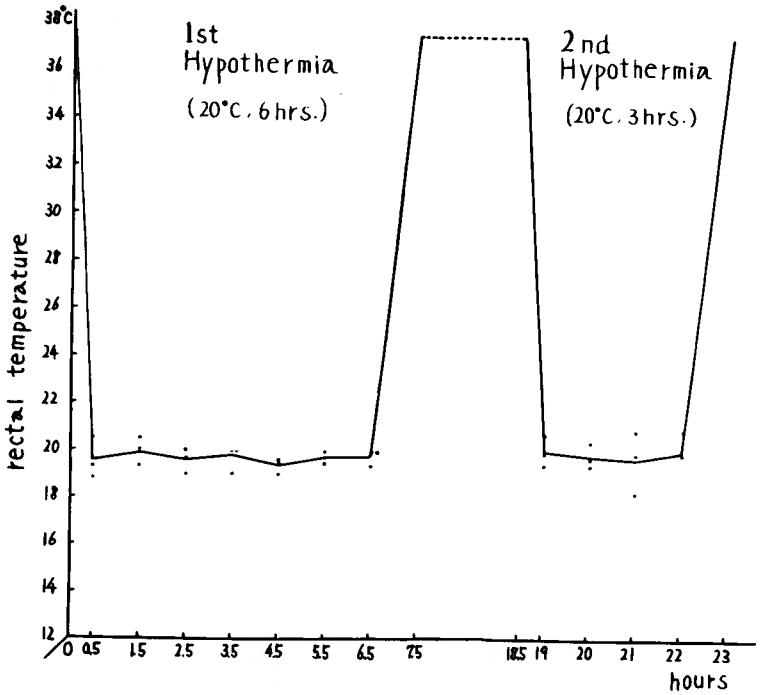

Fig. 2 Rectal temperature of mice subjected to repeated hypothermia at 20°C, for six hours and three hours

during the second hypothermia being kept at 20°C for three hours. In the third group (Fig. 3), mice were cooled so that the rectal temperature might be lowered to the level of 15°C at an interval of one hour after the beginning of hypothermia. The body temperature of the cooled mice were kept at the same level of 15°C for six hours. In each group, 25mg of additional Nembutal per kilogram of body weight, was injected during hypothermia, when necessary, in order to have the mice remain quietly on the tray. After maintaining a given level of hypothermia for a certain period, the mice were rewarmed by heating the tray from outside, so that the body temperature might be restored to normal at an interval of one hour after being released from hypothermia.

Ascitic fluid was aspirated from each mouse every two hours during and after hypothermia to make smears for cytological examination. The smears were dryed and fixed by flooding at once with methanol. The hydrolysis by heat was performed at 60°C for 10 minutes and washed for 2 minutes in running tap water. The smears were exposed to Feulgen reagent at room temperature for 30 minutes. They were placed in 3 changes of bleaching solution, 3 minutes in each. After being washed, the smears were stained with 2 per cent Giemsa's solution for ten minutes, and rinsed. With the Feulgen-Giemsa staining, the nucleus appears violet and cytoplasm pink in color.

The smears were examined to count cancer cells in mitosis under magnification with a 100× oil-immersion objective and 10× eyepiece. In each smear preparation, two thousand tumor cells were examined. A mean value of mitotic indices of the smears which were made simultaneously from three mice per group was presented as a mitotic

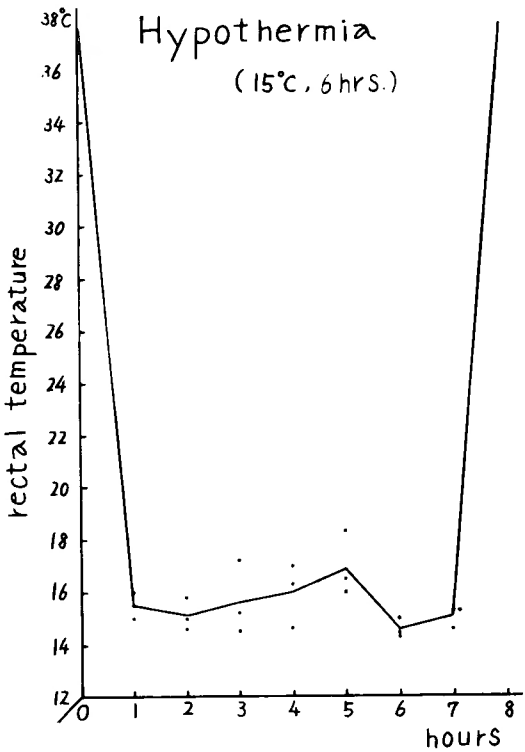

Fig. 3 Rectal temperature of mice subjected to hypothermia at 15°C for six hours

index of a given group. Mitotic indices of cells found in different phases of mitosis were also calculated. The most conspicuous characteristics of cells in prophase are condensation of the chromosomes, disappearance of the nucleus and dissolution of the nuclear membrane. Metaphase is characterized by complete disappearance of the nuclear membrane and gradual movement of chromosomes which arrange themselves along the equatorial plate. The parting of sister chromosomes which move from the plate toward the pole, is a distinct event making the transition from metaphase to anaphase. The main events in telophase are reformation of nucleoli, reestablishment of nuclear membrane and dismantling of the mitotic apparatus.

In addition to the experimental mice mentioned above, three control mice which were bearing the tumor were used: two for studying the difference in body temperatures at various body sites during hypothermia and the other one for examining the influence of Nembutal anesthesia per se on mitotic index.

RESULTS

1) Difference in body temperature measured at various sites of a cooled mouse

A control mouse was submitted to hypothermia at 20°C and the other one at 15°C. About one hour after beginning the hypothermia, i. e. in an early stage of maintenance of the hypothermia, body temperature was measured at various sites of the body in the

same way as in the rectum. The results are given in Tables I and II. The difference between the rectal temperature and that in the abdominal cavity was not greater than 1°C. The difference between the skin temperature on the abdomen and that in the abdominal cavity was less than 2°C.

The average normal rectal temperature of ten mice was 37.6°C with a range of 37.0°C to 38.2°C in the environment at 23°C.

Table I

rectal temp.	temp. in abdominal cavity	skin temperature on									
		head	ear	nose	neck	fore leg	back	chest	abdomen	hing leg	tail
20.0	20.5	19.8	17.6	17.5	21.0	15.2	21.5	19.6	19.6	14.5	14.4

Body temperature measured at various sites in the early stage of maintenance of hypothermia at 20.0°C

Table II

rectal temp.	temp. in abdominal cavity	skin temperature on									
		head	ear	nose	neck	fore leg	back	chest	abdomen	hind leg	tail
15.0	15.7	14.6	15.0	13.6	14.9	13.6	15.8	14.0	14.0	13.3	13.2

Body temperature measured at various sites in the early stage of maintenance of hypothermia at 15.0°C

2) Influence of Nembutal anesthesia on mitotic index (Fig. 4)

Another control mouse, four days after transplantation of 15 million cells, was injected with 50mg of Nembutal per kilogram of body weight. When the mouse was kept in an environment at 28°C, its rectal temperature remained at the level of normal body temperature. The mitotic index in the ascitic fluid from the mouse, being 1.9% before injection

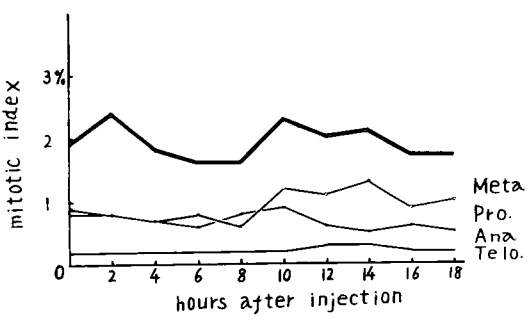

Fig. 4 Mitotic counts of Ehrlich ascites cancer cells in the mouse injected with 50mg of Nembutal per kilogram of body weight
Thick line : mitotic index of cells in mitosis.
Pro. : mitotic index of cells in prophase.
Meta. : mitotic index of cells in metaphase.
Ana. Telo. : mitotic index of cells in anaphase-telophase.

of Nembutal, remained almost at the same level for at least a period of about 18 hours, ranging from 1.6% to 2.4% under anesthesia.

3) Changes in mitotic index after hypothermia

a) Six hours of hypothermia at 20°C (Fig. 5, and Photos. 1, 2 and 3)

The mitotic index which was 1.7% before hypothermia, was markedly reduced during hypothermia; 1.4%, 0.7% and 0.7%, two and a half, four and a half, and six and a half hours after the beginning of hypothermia respectively. After rewarming, the mitotic index increased rapidly. Four hours after rewarming, the curve of mitotic index reached the first peak, which gradually decreased thereafter. Twelve hours after rewarming, the mitotic index reached the lowest level of 0.8%. Eighteen hours after rewarming the second peak was observed. The interval between the two peaks was 14 hours. Before hypothermia, the mitotic index was 1.7%; the mitotic index of cells found in prophase was 0.7%, 0.8% in metaphase, and 0.2% in anaphase-telophase. At the first peak, the mitotic index was 3.6%; 0.9% in prophase, 2.2% in metaphase, and 0.5% in anaphase-telophase. The increase in mitotic index at the first peak was mainly caused by that in metaphase index. At the second peak, the mitotic index was 3.2%; 1.5% in prophase, 1.5% in metaphase, and 0.2% in anaphase-telophase.

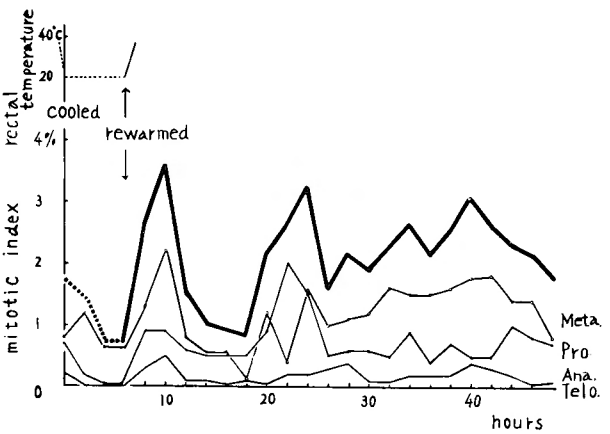

Fig. 5 Mitotic counts in Ehrlich ascites cancer cells during and after six hours of hypothermia at 20°C
Thick line : mitotic index of cells in mitosis.
Pro. : mitotic index of cells in prophase.
Meta. : mitotic index of cells in metaphase.
Ana. Telo. : mitotic index of cells in anaphase-telophase.

b) Repeated hypothermia at 20°C (Fig. 6, and Photos. 4, 5 and 6)

The mitotic index before hypothermia was 2.1% in this group; 0.8% in prophase, 1.1% in metaphase, and 0.2% in anaphase-telophase. Immediately after the first hypothermia at 20°C for six hours, the mitotic index was 1.1%; 0.1% in prophase, 1.0% in metaphase, and 0.05% in anaphase-telophase. The first peak of the mitotic curve was observed two hours after the first rewarming. The mitotic index at the first peak was 3.1%; 1.2% in prophase, 1.3% in metaphase, and 0.6% in anaphase-telophase. The mitotic index gradually decreased after the first peak. Twelve hours after the first rewarm-

ing, the mice were rechilled at 20°C for three hours. Immediately after the second hypothermia, the mitotic index was 1.2% ; 0.1% in prophase, 1.1% in metaphase, and 0.05% in anaphase-telophase. Eight hours after the second rewarming, the mitotic index reached a high level at 5.5% ; 2.4% in prophase, 2.5% in metaphase, and 0.6% in anaphase-telophase, and then gradually declined.

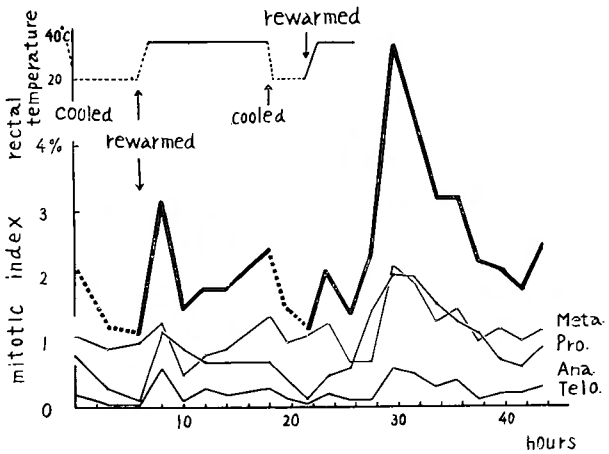

Fig. 6 Mitotic counts in Ehrlich ascites cancer cells during and after repeated hypothermia at 20°C, for six hours and three hours
Thick line : mitotic index of cells in mitosis.
Pro. : mitotic index of cells in prophase.
Meta. : mitotic index of cells in metaphase.
Ana. Telo. : mitotic index of cells in anaphase-telophase.

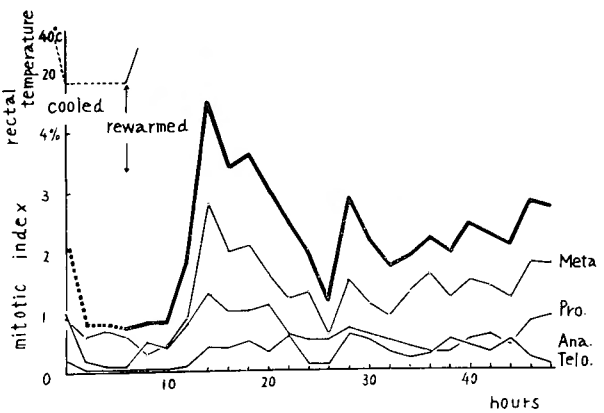

Fig. 7 Mitotic counts in Ehrlich ascites cancer cells during and after six hours of hypothermia at 15°C
Thick line : mitotic index of cells in prophase.
Pro. : mitotic index of cells in prophase.
Meta. : mitotic index of cells in metaphase.
Ana. Telo. : mitotic index of cells in anaphase-telophase.

c) Six hours of hypothermia at 15°C (Fig. 7, and Photos. 7, 8 and 9)

The mitotic index before hypothermia was 2.1% in this group; 1.0% in prophase, 0.9% in metaphase, and 0.2% in anaphase-telophase. The mitotic index declined to 0.8%, 0.8% and 0.7%, three, five and seven hours after beginning of hypothermia respectively. Eight hours after rewarming, the curve of mitotic index reached the first peak, which gradually decreased thereafter. At the first peak, the mitotic index was 4.5%; 1.3% in prophase, 2.8% in metaphase, and 0.4% in anaphase-telophase. Twenty hours after rewarming, the mitotic index reached the lowest level of 1.2%, and twenty two hours after rewarming, the second peak was observed. At the second peak, the mitotic index was 2.8%; 0.7% in prophase, 1.5% in metaphase, 0.6% in anaphase-telophase. The interval between the two peaks was 14 hours

DISCUSSION

In nature, there are some organisms in which cells divide synchronously without any artificial alteration of their environments. For example, sea urchin eggs, epitherial cells in the cornea and duodenum of mice, and epidermal cells in both mice and human beings have been reported to show the diurnal periodicity in the occurrence of mitosis[10)14)40)41)]. On trying to explain these phenomena, MAZIA proposed a conception of "natural rhythm", and suggested that in higher plants and animals this might be the factor linking the mitotic rhythms to phisiological rhythms affecting the cell's environment[23)]. On the other hand, there are many reports on synchronous mitotic rhythms artificially produced by some means [7)9)19)22)26)30)31)34)35)36)], for which MAZIA proposed the term "experimental synchronization". For example, SCHERBAUM and ZEUTEN induced an experimental synchronization in Tetrahymena pyriformis[36)]. By changing culture-temperature from 28°C to a sublethal temperature at 34°C every 30 minutes, the cells were shocked interruptedly for 7 hours. During the repeated heat shock, the cells were thought to be arrested, set back in regard to mitotic process and finally caught in the same stage of interdivision period. Thereafter, three successive peaks of the mitotic index were observed, which were followed by an actual increase in cell number by 85, 83 and 64 per cent respectively. The intervals between these peaks were shorter than the normal generation time.

NEWTON and WILDY succeeded in inducing in-vitro synchronization of HeLa cells with one temperature shock[26)]. HeLa cells which were grown at 37°C for 24 hours were exposed to 4°C for one hour. Upon return to 37°C there was no cell division for 17 hours, then as many as 95% of the cells divided within one hour thereafter. The second burst of cell division followed within 18 hours. A rapid increase in mitotic index was observed to precede the actual increase in cell number. In their experiments in which 60% to 80% of the cells divided within an hour or less, the maximum mitotic index after temperature shock ranged from 3.5% to 8%, while the normal mitotic index was 1.7%. Judging from the actual increase in cell number, it was clear that a markedly high degree of synchrony in cell division was achieved. In a group of our experiment, the maximum mitotic index in the ascites after hypothermia was as high as 5.5%, while the normal mitotic index was 2.1%. These findings, as in the case of NEWTON and Wildy's observations on temperature-induced synchronous cell division, might leave no doubt that the peaks

of mitotic index observed in this study were followed by mitosis, resulting in an actual increase in cell number. In such cases, the rate of increase in cell number is thought to be greater than what is impressed by the changes in mitotic index; the number of cells with mitotic figures at any time preceding division seems to be rather small. This apparent discrepancy between increases in cell number and changes in mitotic index might be due to different ways of measurement; momentary measure of mitotic index and hourly observation of cell number. CAMPBELL suggested that the degree of synchrony could be expressed as changes in cell number on the one hand or in terms of mitotic index on the other[5]. In our case of Ehrlich ascites cancer cells, the degree of synchrony of mitosis might be expressed in a more concrete form when an actual increase in cell number was measured.

To obtain a higher degree of synchronous mitosis, cells in a greater number need to be arrested and set back in regard to mitotic process. As far as temperature shock is concerned, a higher degree of synchronization of cell division can be expected by changing the condition of cold shock in the following three ways; lower temperature, longer duration of hypothermia and repetition of hypothermia.

In our experiment, the peak of mitotic index after hypothermia was higher in the group of hypothermia of 15°C than in that of 20°C. EVANS and SAVAGE showed that the lower the culture-temperature, the longer was the mitotic time in Vicia faba, and that its division was almost arrested at 3°C[12]. NIAZI and LEWIS, on the other hand, administered profound hypothermia of −4°C to 8.5°C to 21 rats, 13 of which recovered from the hypothermia and survived well[27]. If mice which were bearing Ehrlich ascites cancer were cooled to such a degree as in their experiments, cell division might be synchronized to a higher degree.

SCHERBAUM and ZEUTEN observed that six to ten hours of repeated heat shocks were most effective to induce a high degree of synchronous division in mass cultures of Tetrahymena pyriformis, and proved that the treatment of a shorter duration tended to produce lower peaks of division activity[36]. According to KATO, when mice which were bearing Sarcoma 180 were cooled at 20°C for two, six or ten hours, the peak of mitotic index in the tumor cooled for ten hours was the highest[19]. These results seem to indicate that, so far as synchronous mitosis produced by hypothermia is concerned, the longer the duration of hypothermia, the higher the degree of synchrony of mitosis.

NEWTON and WILDY observed that when HeLa cells were rechilled 14 or more hours after the initial cold shock, the effect delayed the burst of division by about one hour. When, however, the cells were rechilled less than 14 hours after the initial cold shock, the time of synchronized division was delayed for a further 18 hours[26]. These results seem to indicate that after the initial cold shock there was an appropriate time for the second cold shock to delay the burst of cell division. In general, when the burst of cell division is delayed by means of such procedures as the second cold shock, so many cells are thought to be synchronized. HAMBURGER and ZEUTEN observed that delay of division of Tetrahymena pyriformis synchronized by heat shocks was effected by exposure to dinitrophenol, which inhibited oxidative phosphorylation in the cells. The delay was proportional to the interval between the end of heat shock and time of beginning the exposure

to dinitrophenol. The maximum delay was obtained when the cells were exposed to dinitrophenol 25 minutes before a given division[16]. The time of the most effective exposure falls on about the two-third point of the interval between the release of heat treatment and the first peak of mitotic index. In our experiment of repeated hypothermia, although the appropriate time for the second hypothermia was not yet determined, at the two-third point of the interval between the first and second peaks of mitotic curve i. e. 12 hours after the first rewarming, the mice were rechilled. Eight hours after the second rewarming, the peak of mitotic index had a value of 5.5%, which was definitely higher than the peak of mitotic index produced by the single hypothermia.

According to BASERGA, the generation time determined by autoradiography was 18 hours in Ehrlich ascites cancer cells[2]. In the present experiment, the interval between the two successive peaks in the mitotic curve observed after hypothermia, was shorter than the generation time reported by BASERGA or KLEIN and RÉVÉSZ[2)20)]. While the generation time of Tetrahymena pyriformis was 135 minutes, the interval between the two successive peaks which were observed after heat shock in SCHERBAUM and ZEUTEN's experiment, was 100 minutes[36]. As for HeLa cells, the generation time was 26 hours, whereas the interval between the two successive peaks produced by cold shock in NEWTON and WILDY's experiments was 18 hours[26]. All these intervals were approximately two thirds of the normal generation time. The reason why the peaks appear to be closer in time than indicated by the duration of the normal mitotic cycle has not been elucidated yet. In the present experiment, the interval between the the two peaks was 14 hours both after hypothermia of 20°C for six hours and after hypothermia of 15°C for six hours. The fact that the interval was the same, independent of the degree of temperature shift, was also observed in HeLa cells by NEWTON and WILDY, who showed that the interval in HeLa cells was 18 hours after a single cold shock as well as repeated cold shocks, while the generation time 26 hours[26].

PART II

INTENSIFICATION OF ANTICANCER EFFECT OF ENDOXAN BY USE OF HYPOTHERMIA

With the same strain of mice and the same experimental tumor as in Part I, influence of hypothermia on the effect of a radiomimetic anticancer agent was examined.

Mice were inoculated intraperitoneally with two million cells of Ehrlich ascites cancer. Four days after the inoculation the mice were divided into several experimental groups according to the degrees of hypothermia and the periods of administration of anticancer agent which were to be given to the mice. After being anesthetized with Nembutal, the mice were subjected to hypothermia for a given period and then rewarmed, in the same way as described in Part I. Endoxan in 0.2cc of physiological saline solution was administered intraperitoneally to the mice at a dose level of 50mg per kilogram body weight once at given time after hypothermia. In some mice, the drug was given during hypothermia. The effect of the drug on each group was compared by measuring survival days of the animal. Each group comprised twelve to fifteen tumor bearing mice, some of which died during and just after hypothermia. These mice which could not tolerate the

hypothermia were excluded from calculating survival days. In addition to the experimental groups described above, several control groups of mice which were bearing the same tumor were examined to determine the effects of hypothermia or administration of Endoxan alone on the survival days of animals.

Experiment I Six hours of hypothermia at 20°C (Figs. 8-1 and 8-2)

Group I (Without hypothermia or the administration of Endoxan)

Ten tumor-bearing mice were examined, to which neither hypothermia nor administration of Endoxan was performed.

Two mice died on the 14th day, three on the 16th day, and five on the 17th day after tumor inoculation, respectively.

Group II (Administration of Endoxan alone)

Six mice, without being subjected to hypothermia, received an intraperitoneal injection of Endoxan.

One mouse died on the 11th day, four on the 16th day, and one on the 17th day after tumor inoculation, respectively.

Group III (Hypothermia alone)

Fourteen mice were subjected to six hours of hypothermia at 20°C alone without administration of Endoxan.

One mouse died on the 13th day, two on the 15th day, five on the 16th day, and six on the 17th day after tumor inoculation respectively.

Group IV (Administration of Endoxan during hypothermia)

Fifteen mice were subjected to the same hypothermia as in Group III and injected with Endoxan two and a half hours after the beginning of hypothermia.

One mouse died on the 12th day, one on the 14th day, three on the 16th day, five on the 17th day, two on the 19th day, and three on the 21st day after tumor inoculation, respectively.

Group V (Administration of Endoxan immediately after rewarming)

Six mice were subjected to the same hypothermia and injected with Endoxan immediately after rewarming.

Two mice died on the 17th day, and four on the 19th day after tumor inoculation.

Group VI (Administration of Endoxan one hour after rewarming)

Seven mice were subjected to the same hypothermia and injected with Endoxan one hour after rewarming.

Three mice died on the 17th day, one on

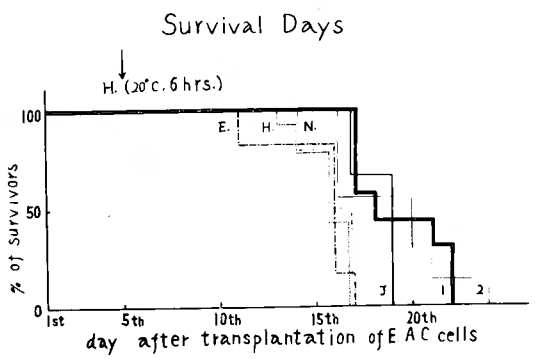

Fig. 8-1 Survival days of mice treated with Endoxan after 6 hours of hypothermia at 20°C

E. : Group II, treated with Endoxan alone.

H. : Group III, subjected to hypothermia (20°C, 6hrs.) alone.

N. : Group I, not treated.

J. : Group V, treated with Endoxan immediately after hypothermia (20°C, 6hrs.).

1 : Group VI, treated with Endoxan one hour after hypothermia (20°C, 6hrs.).

2 : Group VII, treated with Endoxan two hours after hypothermia (20°C, 6hrs.).

H. (20°C, 6hrs.) : hypothermia at 20°C for 6 hours.

the 18th day, one on the 21st day, and two on the 22nd day after tumor inoculation, respectively.

Group VII (Administration of Endoxan two hours after rewraming)

Seven mice were subjected to the same hypothermia and injected with Endoxan two hours after rewarming.

Three mice died on the 16th day, two on the 20th day, one on the 21st day, and one on the 24th day after tumor inoculation, respectively.

In Groups VI and VII, the survival days seemed to be prolonged slightly as compared with Groups I, II and III.

Experiment II Repeated hypothermia at 20°C (Fig. 9)

Group I (Repeated hypothermia alone)

Seven mice, which were subjected to a rectal temperature at 20°C for six hours, were rewarmed and twelve hours thereafter rechilled at 20°C for three hours. Administration of Endoxan was not performed. One mouse died on the 8th day, two on the 15th day, and four on the 18th day after tumor inoculation, respectively.

Group II (Administration of Endoxan after the second hypothermia)

Seven mice were subjected to repeated hypothermia in the same way as in Group I and injected with Endoxan three hours after the second rewarming.

Four mice died on the 22nd day, two on the 24th day, and one on the 25th day after tumor inoculation, respectively.

In Group II, the survival days were definitely prolonged as compared with those of the control groups in Experiment I and II.

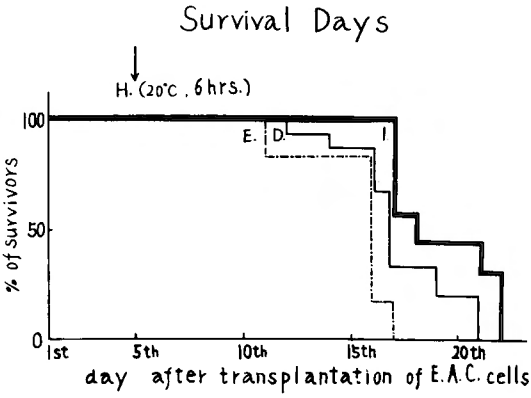

Fig. 8-2 Survival days of mice treated with Endoxan after 6 hours of hypothermia at 20°C
E. : Group II, treated with Endoxan alone.
D. : Group IV, treated with Endoxan during hypothermia (20°C, 6hrs.).
1 : Group VI, treated with Endoxan one hour after hypothermia (20°C, 6hrs.).
H. (20°C, 6hrs.) : hypothermia at 20°C for 6 hours.

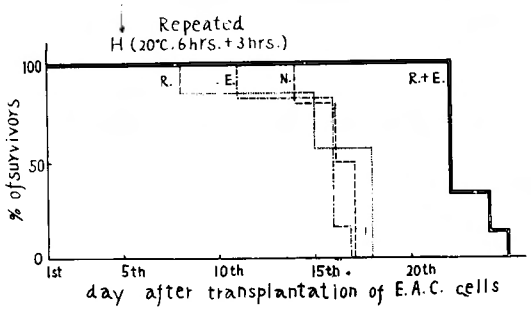

Fig. 9 Survival days of mice treated with Endoxan after repeated hypothermia
R. : Group I. subjected to repeated hypothermia alone.
E. : Group II in Exp. I, treated with Endoxan alone.
N. : Group I in Exp. I, not treated.
R.+E. : Group II, treated with Endoxan after repeated hypothermia.
Rpeated H. (20°C, 6hrs.+3hrs.) : repeated hypothermia at 20°C for 6 hours and 3 hours.

Experiment III Six hours of hypothermia at 15°C (Fig. 10)

Group I (Hypothermia alone)

Five mice were subjected to six hours of hypothermia at 15°C alone. Four mice died on the 15th day, and one on the 16th day after tumor inoculation.

Group II (Administration of Endoxan after hypothermia)

Four mice were subjected to the same hypothermia as in Group I and injected with Endoxan three hours after rewarming.
One mouse died on the 19th day, one on the 22nd day, one on the 28th day, and one on the 30th day after tumor inoculation, respectively.

In Group II, the survival days were definitely prolonged as compared with those of the control groups in Experiment I and III. Some mice survived much longer than the mice in Group II of Experiment II.

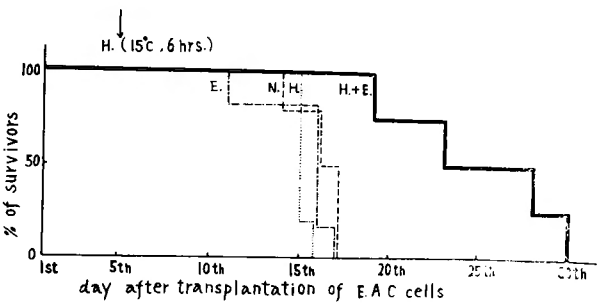

Fig. 10 Survival days of mice treated with Endoxan after 6 hours of hypothermia at 15°C
E. : Group II in Exp. I, treated with Endoxan alone.
N. : Group I in Exp. I, not treated.
H. : Group I, subjected to hypothermia (15°C, 6hrs.) alone.
H.+E. : Group II, treated with Endoxan after hypothermia (15°C, 6hrs.).
H. (15°C, 6hrs.) : hypothermia at 15°C for 6 hours.

DISCUSSION

ARNOLD and co-workers in 1957 synthesized Endoxan, a cyclic phosphamide ester of nitrogen mustard[1]. To be activated in vivo, Endoxan needs phosphamidase or phosphatase, which breaks the cycle group at the phosphorous nitrogen or the phosphorous-oxygen bonding[8]. By histochemical methods, phosphamidase is demonstrated in an increased amount in cancerous tissue, which is a possible selective site of action for this agent. MCDONALD et al., using Ehrlich ascites carcinoma, Krebs ascites carcinoma and Andrervont breast carcinoma, showed that alkylating agents such as Endoxan inhibited DNA synthesis at the stage of incorporation, i. e. thymidine into DNA[24]. In general, it can be said that an alkylating agent is the most effective in dividing cells when it is given to the cells in the period of DNA synthesis[4)13)29)].

Many investigators who studied the relation of time course between DNA synthesis and cell division by photometric or tracer method, demonstrated that DNA was synthesized during interphase, most vigorously in late interphase[11)23)32)]. In synchronized cell populations, morphological synchronization of mitosis was found to be in accord with synchrony of DNA synthesis. For example, in cultures of HeLa cells a burst of DNA synthesis was observed to precede synchronized mitosis[26)38)]. Similar observations were reported in synchronized Tetrahymena pyriformis[35)]. BLUM and PADILLA showed a high degree of synchrony of DNA, RNA and protein synthesis in synchronized Astasia longa[3)]. These reports indicated that in synchronized cell populations most DNA was synthesized also in late

interphase. TAKAHASHI observed that, when the body temperature of NF sarcoma-bearing mice subjected to six hours of hypothermia at 20°C were returned to the normal body temperature, the dark-reaction in DNA autoradiograms made from the tumor tissue was maximum in the period between two to four hours after rewarming[42]. In our experiment, when the tumor-bearing mice were subjected to hypothermia at 15°C for six hours or repeated hypothermia at 20°C, the first peak of mitotic index appeared eight hours after rewarming. Five hours before the peak of mitotic index Endoxan was administered to the mice. The time of administration of the agent was thought to fall on the late interphase of the synchronized cell populations.

Influence of hypothermia or hyperthermia upon tumor growth has been studied by many investigators[15)18)21)28)37)39)43]. The first clinical use of hypothermia was reported by SMITH and FAY in 1939[39]. With the intention of controlling tumor growth, they administered hypothermia to patients with malignancy. Improvement of objective findings, however, was not observed. The other attempts to treat cancer using hypothermia itself have not given satisfactory results[15)18)28]. In regard to the influence of low temperature on metabolism of cancer cells, there are many reports. For example, MORGAN observed the effect of low temperature on ascites tumor cells; the tumor cells of 6C3HED lymphosarcoma, TA3 mammary carcinoma and Ehrlich ascites cancer, after being stored frozed at −70°C for 6 months, remained viable[25]. CASSEL reported that after being preserved at 4°C for 7 days, 91.9% of Ehrlich ascites cancer cells was viable when examined by Schreck's viability count test[6]. PATTERSON showed that during hibernation of hamster inoculated with a human cancer, tumor growth was markedly inhibited and resumed its growth when the animal was returned to a warm room temperature[28]. These reports indicate that hypothermia, so far as these experiments are concerned, did not effect severe irreversible degeneration of tumor cells. This is concordant with our observations that the tumor cells subjected to hypothermia did not show any sign of degeneration when stained by Feulgen-Giemsa method, and that the survival days of our control animals subjected to hypothermia were proved to be almost the same as those of the control group without hypothermia.

According to Hornsey, mice irradiated with a large doses of 1500r. (LD_{50} 620r.) under hypothermia of 1°C to 0°C survived for as long as thirty days[17]. The cooled mice seem to possess some protection against irradiation. Under hypothermia, animals might be expected to have the same protection against alkylating agents as against irradiation. If so, side effects of the agents could be reduced, when the agents are administered under hypothermia. At the same time, however, the anticancer effect of the agents might be reduced. In our animals, which were injected with Endoxan during hypothermia, the survival days seemed to be almost the same as those in the control groups.

Since the rate of reaction of most metabolic or enzymatic processes is thought to be decreased or increased to about one third of normal value for each 10°C, it can be assumed that the effect of any anticancer agent upon tumor cells might be increased in a hyperthermic environment[33]. In the field of perfusion therapy for malignant tumors, local hyperthermia has been used to intensify the effects of anticancer agents[37)43]. In our experiment, the body temperature of the mice which were subjected to hypothermia returned

to normal before the administration of anticancer agent. When an appropriate hyperthermia is administered after the hypothermia, the effect of Endoxan might be intensified more than was seen in our present study. The hyperthermia might also effect a higher degree of synchronization to tumor cells, which would result in better effects of anticancer agents.

SUMMARY

In-vivo synchronous mitosis of cancer cells was studied in mice inoculated intraperitoneally with Ehrlich ascites cancer cells. The mice were subjected to hypothermia of varying degrees, and changes in mitotic index in the ascites during and after the hypothermia were examined. After being released from the hypothermia, the mice were injected with a single dose of Endoxan, the effect of which was compared with that in the control groups.

The results were as follows:

1) When the mice were cooled at 20°C for six hours, the first peak of mitotic index, at a value of 3.6%, appeared four hours after rewarming, followed by the second peak of 3.4% fourteen hours later.

2) When the mice were subjected to repeated hypothermia at 20°C for six hours and three hours, a peak of mitotic index, at a value of 5.5%, appeared eight hours after the second rewarming.

3) When the mice were cooled at 15°C for six hours, the first peak of mitotic index, at a value of 4.5%, appeared eight hours after rewarming, followed by the second peak of 2.8% fourteen hours later.

4) When the mice were injected with Endoxan three hours after being released from hypothermia of a duration of 6 hours at 15°C or repeated hypothermia of a duration of 9 hours in total at 20°C, their survival days were markedly prolonged as compared with those of the control animals which were treated with Endoxan alone.

I wish to express my sincere gratitude to Assistant Professor Ikuzo Yokoyama for his friendly guidance throughout the course of the work.

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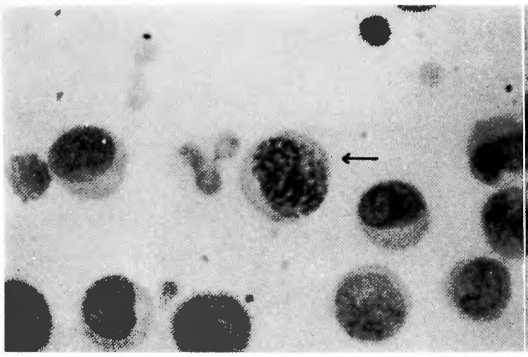

Photo. 1 Ehrlich ascites cancer cell with prophasic nucleus (arrow) before six hours of hypothermia at 20°C

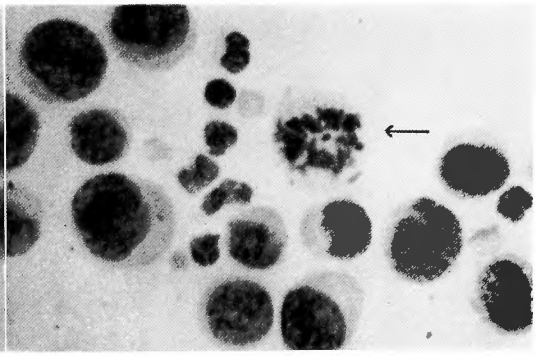

Photo. 2 Ehrlich ascites cancer cell with metaphasic nucleus (arrow) at the end of six hours of hypothermia at 20°C

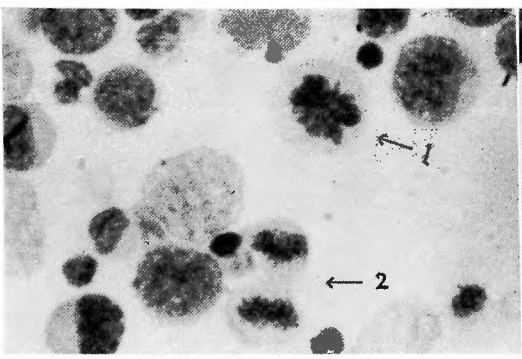

Photo. 3 Ehrlich ascites cancer cells with metaphasic nucleus (arrow 1) and with telophasic nucleus (arrow2), four hours after rewarming from six hours of hypothermia at 20°C

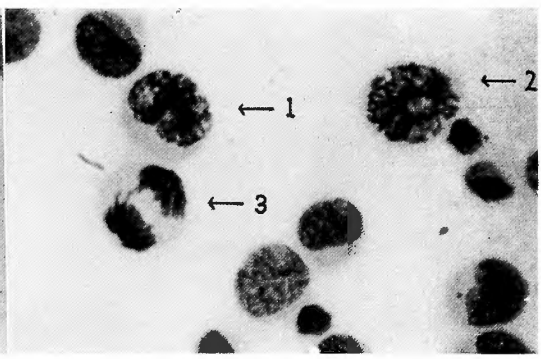

Photo. 4 Ehrlich ascites cancer cells with prophasic nucleus (arrow 1), metaphasic nucleus (arrow 2) and anaphasic nucleus (arrow 3), before repeated hypothermia at 20°C, for six hours and three hours

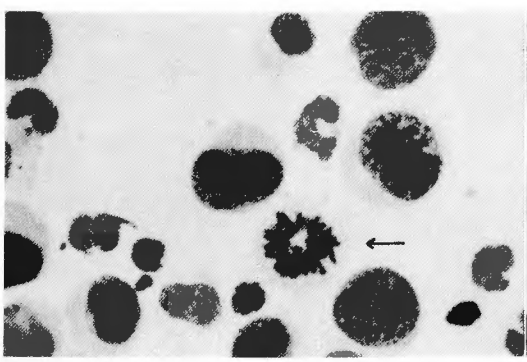

Photo. 5 Ehrlich ascites cancer cell with metaphasic nucleus (arrow) at the end of the second hypothermia at 20°C for three hours

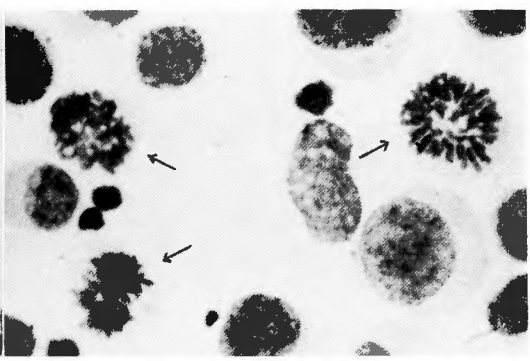

Photo. 6 Ehrlich ascites cancer cells with metaphasic nuclei (arrows), eight hours after the second rewarming from repeated hypothermia at 20°C, six hours and three hours

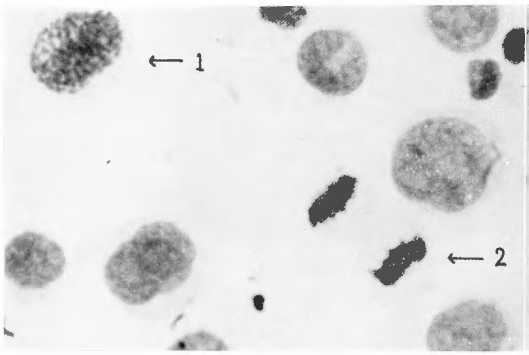

Photo. 7 Ehrlich ascites cancer cells with prophasic nucleus (arrow 1) and telophasic nucleus (arrow 2), before six hours of hypothermia at 15°C

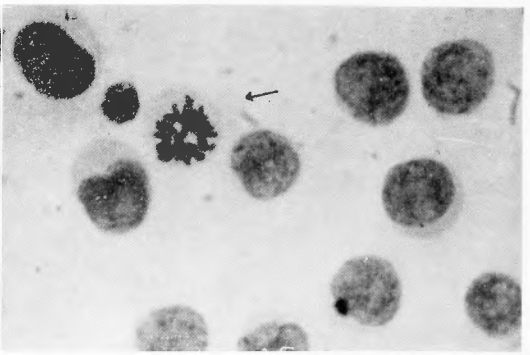

Photo. 8 Ehrlich ascites cancer cell with metaphasic nucleus (arrow) at the end of six hours of hypothermia at 15°C

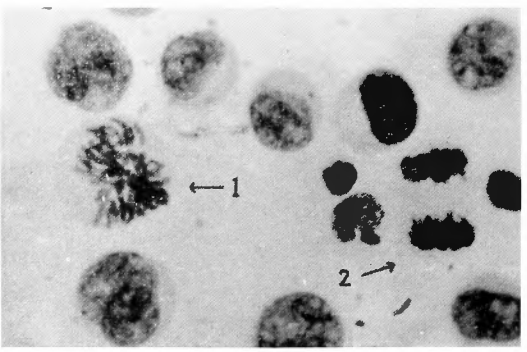

Photo. 9 Ehrlich ascites cancer cells with metaphasic nucleus (arrow 1) and anaphasic nucleus (arrow 2), eight hours after rewarming from six hours of hypothermia at 15°C

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和文抄録

低体温法による腫瘍細胞の生体内同調分裂及び癌化学療法への応用

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制癌剤は個々の腫瘍細胞の分裂週期のうちの或るstadiumに特に鋭敏に作用するといわれている．もしat randomに分裂している腫瘍細胞の分裂を同調させる事が出来れば，制癌剤をその最も鋭敏な時期に与える事によつて，治療効果を増強する事が可能と思われる．細胞分裂を同調させる方法として，低体温法を利用した．まず，低体温法が腫瘍細胞の分裂を生体内で同調させる事が出来るかどうかを形態学的に検索するため，エールリッヒ腹水癌細胞1,500万個を腹腔内に移植した4日目のマウスに，一定の条件の低体温を施行して低体温中並びに復温後に於ける腫瘍細胞の分裂指数の変動を組織学的に検索した．又，低体温の温度，持続時間，頻度を変える事によつて，腫瘍細胞の分裂同調の程度に，いかなる影響を及ぼすかを検査した．更に担癌マウスに一定の条件の低体温法を施し，復温後，同調的細胞分裂のおこる前のDNA合成期を狙つて，DNA合成を阻害する制癌剤Endoxanを投与して，制癌効果が増強されるかどうかを検索した．その成績は次の如くである．

1) 担癌マウスに20℃6時間の低体温法を施行した場合の腫瘍細胞の分裂指数曲線の変遷については，常体温時1.7%の指数値は低体温により0.8%にまで低下したが，復温と同時に急増して，復温後4時間目には3.6%の高値に達し，以後急減した．その後再び増加して復温後18時間目に3.2%の第2の高値が出現した．

2) 担癌マウスに20℃6時間の低体温法を施行し復温後12時間目に再び20℃3時間の低体温法を施行した場合には，常体温時2.1%の指数値は第1回の低体温により1.1%にまで低下し，復温と同時に急増して復温後2時間目に3.1%の高値に達した．以後急減して第2回の低体温により1.2%にまで低下し第2回目の復温後8時間目に再び5.5%の高値に達した．

3) 担癌マウスに15℃6時間の低体温法を施行した場合には常体温時2.1%の分裂指数は低体温により0.7%にまで低下し，復温後上昇して8時間目に4.5%の高値に達した．その後低下し，復温後22時間目に再び2.8%の高値に達した．

4) 細胞分裂の同調化の程度は，20℃6時間の1回の低体温法の場合に比較して，20℃6時間及び3時間の2回低体温法又は15℃6時間の低体温法の場合が優れていた．即ち，我々の実験した範囲内では，低体温の温度を低くし且つ持続時間を長くすれば，それだけ腫瘍細胞分裂の同調化の程度が高まる事が判明した．

5) 担癌マウスに15℃6時間の低体温法或いは20℃6時間及び3時間の2回低体温法を施行した後正常体温に復温し，復温後3時間目にEndoxanを1回だけ腹腔内に投与した実験群は，低体温を行なわずに薬剤の投与のみを行なつた対照群に比べて著明な延命効果を認める事が出来た．

6) 以上を要約すると，担癌動物に一定の条件の低体温法を施行した後に正常体温に復温する事により，担癌個体内で腫瘍細胞の分裂を同調させる事が出来，且つこの生体内腫瘍細胞分裂同調の現象を利用して，制癌剤の治療効果を増強できる事が判明した．