The Cytological Effect of Chemicals on Tumors, XIII. Phase Optic Observations on Effects of Carzinophilin and Thio-TEPA upon Dividing Cells in Tissue Culture[1)2)]

By

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Recently Makino and his associates have carried out extensive cytological investigations on the action of chemicals upon cell division, especially upon the chromosomes of tumor cells of rats *in vivo* and *in vitro*, with special regard to tumor damage and regression (cf. Makino 1957, 1959). The majority of the former studies, however, have been done with fixed and stained materials. Every sequence of the observed events in those studies was derived from different cells and did not involve a successive series of changes occurring in a single cell under the action of chemicals. It is needful to follow in the living condition the serial changes of cells in response to chemicals, in order to obtain critical information in contrast to the data derived from fixed and stained materials. The present author undertook a phase microscopy study on effects of Carzinophilin and Thio-TEPA, anti-tumor agents, on dividing cells, in a hope to learn the stage which is most sensitive to drugs in the course of cell division.

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Material and method: The cells for the present study were derived from normal and neoplastic tissue culture sources as follows 1) human lung cells of the HL-C strain Nakanishi (Nakanishi *et al.* 1959), and 2) the HeLa strain (Scherer *et al.* 1953). The routine subcultures were made by means of trypsinization (0.2 per cent for 5 minutes) and dispersion with a pipette in TD-40 flasks. Earle's balanced salt solution containing 10 per cent inactivated bovine serum, 0.5 per cent lactalbumin hydrolysate, 0.1 per cent yeast extract and 100 units of penicillin/ml was employed for maintenance of the strains. The medium was usually changed on the 2nd day after subculture.

For experiment, the cell suspension was transferred to a 12×32 mm cover slip placed in a $17 \times 17 \times 38$ mm culture flask. A simple perfusion chamber was made following the idea of Dr. Nakanishi: a paraffin bank was set on an ordinary microscope slide by painting with several strokes of melted paraffin. The cover slip bearing cells was removed from the culture flask, and set up on the slide as prepared above with several drops of the culture medium. Finally the margin of the cover slip was sealed with melted paraffin,

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leaving two holes at opposite corners of the cover slip so that chemicals could be perfused through a glass pipette from the one side and drawn out with a piece of blotting paper from the other. The cells on the 3rd to the 5th day after subculture proved to be most suitable for observation of cell division because of the prevalence of mitotic figures. A cell in an appropriate mitotic stage was selected, and the chemicals prepared as given below were perfused.

Two kinds of anti-tumor agents were used. Carzinophilin (Kyowa) is an antibiotic extracted from *Streptomyces sahachiroi* (Hata *et al.* 1954), and was used for the treatment of HeLa cells. Thio-TEPA (triethylene thiophosphoramide) is an alkylating substance, and was used for the treatment of HL-C strain cells. The drugs were dissolved in the nutrient fluid at various concentrations; 20γ, 15γ, 10γ, and 5γ/ml for Carzinophilin and 2.5 mg, 1 mg, 0.5 mg, 0.1 mg and 0.05 mg/ml for Thio-TEPA.

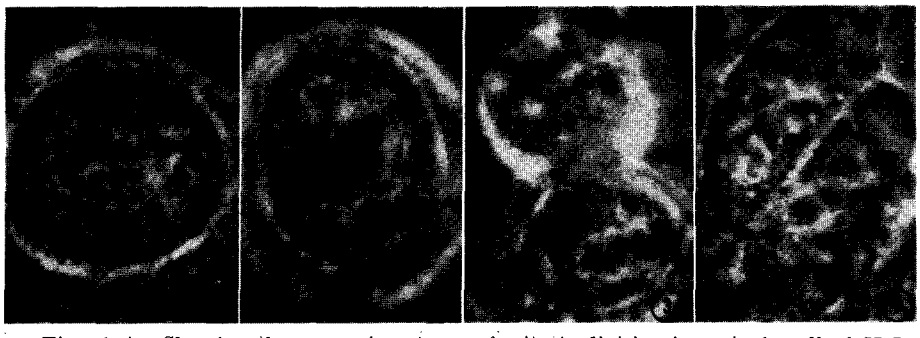
The preparation was kept at 37°C in a thermobox equipped with an Olympus slide type phase-contrast microscope in the optical combination of a 100× objective and an 8× eyepiece, being set up as positve medium phase-contrast.

Observations

1. *Mitotic division of untreated cells*

Both HL-C and HeLa cells were found to follow a nearly similar pattern in the course of division, and therefore the following descriptions are applicable to both.

Prior to any detectable nuclear event at prophase, a retraction of the cytoplasm was observed in the cell. In the early prophase the chromosomes became partially visible, while the nucleoli faded from view. The chromosomes became thicker and



Figs. 1–4. Showing the successive stages of mitotic division in a single cell of HeLa strain, followed from metaphase to interphase. Positive contrast, ×1300. 1, metaphase (taken 104' after preparation). 2, anaphase (141'), 3, telophase (148'). 4, interphase (221').

shorter with time, then the nuclear membrane became irregularly wrinkled and disappeared. Shortly after that disappearance the chromosomes completed their arrangement on the metaphase plate. The area around the metaphase plate remained free from granules. The spindle body seemed to be formed in this area. The spindle body

Table 1. Time relation in cell division of the HeLa strain, untreated.

Cell No.	Prophase	Metaphase	Anaphase	Telophase
1	31min.	54min.	8min.	22min.
2	18	48	6	27
3	22	42	10	38
4	27	46	8	20
5	23	40	11	25
6	31	70	15	42
7	–	48	15	19
8	17	37	13	19
9	27	45	8	26
10	–	57	7	23
11	37	48	11	31
12	–	28	6	28
13	–	32	7	31
14	26	45	6	29
15	24	48	13	26
16	21	41	8	22
17	17	44	9	18
18	21	49	8	21
19	22	47	7	29
20	–	55	7	27
Average	24.27	46.2	9.15	26.15

Table 2. Time relation in cell division of the HL-C strain Nakanishi, untreated.

Cell No.	Prophase	Metaphase	Anaphase	Telophase
1	–min.	31min.	8min.	20min.
2	17	35	8	22
3	–	28	5	33
4	19	37	7	25
5	–	39	6	26
6	21	41	5	23
7	–	48	5	18
8	–	49	11	18
9	–	44	16	27
10	23	32	9	19
11	–	37	9	23
12	–	40	8	26
13	18	30	7	32
14	22	24	9	23
15	–	29	7	24
16	22	25	8	25
17	–	35	10	21
18	20	28	8	22
19	17	25	8	28
20	26	31	6	22
Average	20.5	34.4	8.0	23.9

appeared in a rather spherical form, its longitudinal and transverse axes being approximately alike in length. The separation of the chromosomes and their migration to the opposite poles at anaphase took place almost synchronously for all chromosomes.

Within a few minutes, the cleavage furrow began to appear near the equator of the cell body and cut it into two daughter bodies. When the cytokinesis was completed, the cytoplasmic bubbling began to subside. At the end of telophase the nuclear membrane appeared around the daughter chromosomes. Meanwhile, all visible traces of the chromosomes disappeared from view. The nucleoli could be seen in the nucleus at this time. Finally, the two daughter cells flattened out along the cover slip surface and entered the interphase (Figs. 1–4).

The duration of each mitotic phase was measured in the successive series of a division process followed in the same cell, beginning with prophase or metaphase and ending with telophase. The time of chromosome migration to poles at anaphase was taken as the duration of anaphase. The results of observations are presented in Tables 1 and 2.

2. *Effect of Carzinophilin on division of HeLa cells*

Most of the HeLa cells under the treatment with Carzinophilin showed disintegration in a greater or less degree in the course of cell division, though there were cells which seemed to complete the mitotic cycle. At a dose of 20γ per ml the cells treated at metaphase always showed mitotic abnormalities, and died through the clumping of the chromosomes (Figs. 5–7). At concentrations of 10γ or 15γ per ml, the cells treated at prophase when they entered metaphase underwent pycnotic degeneration of chromosomes. Some metaphasic cells treated at the same dosages as above continued cell division and completed the division. The cells treated at rather later prophase with a dose of 5γ per ml completed division in a regular manner. However, there occurred no advance of the mitotic process in the cells treated in earlier stages of prophase at the same dose as above.

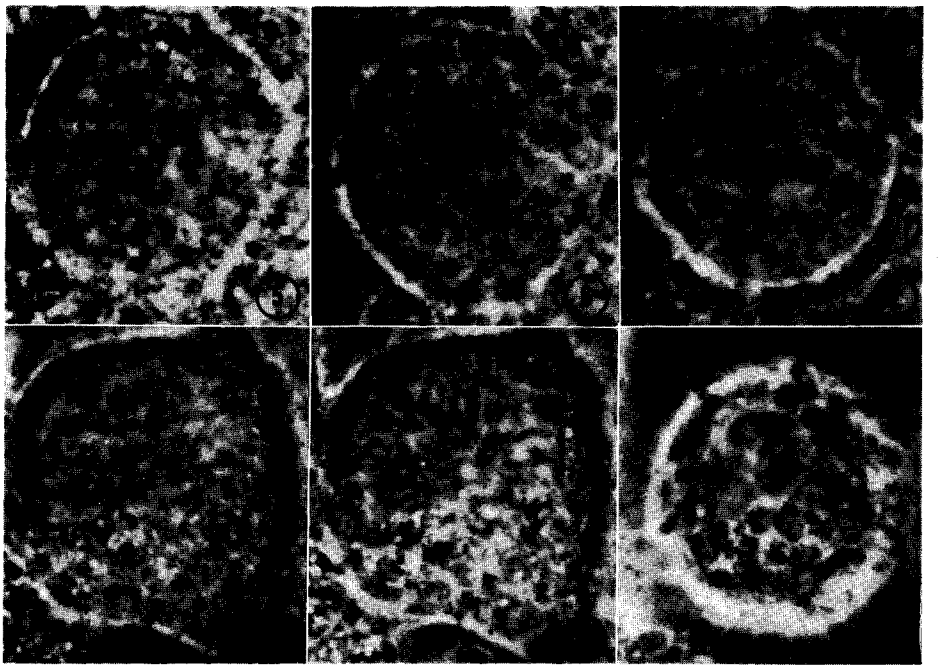
Time relations of each mitotic phase under the influence of Carzinophilin at a dose of 5γ per ml were measured in successive series of a division process followed through single cells, beginning with prophase and ending with telophase. The results are as shown in Table 3.

Table 3. Time relation in cell division of the HeLa strain following the application of Carzinophilin at a dose, 5γ per ml.

Cell No.	Metaphase	Anaphase	Telophase
1	72min.	9min.	22min.
2	54	6	31
3	46	8	26
4	63	15	24
5	57	11	22
6	48	4	29
7	61	10	28
8	58	10	28
9	62	9	26
10	49	8	27
Average	57.0	9.0	26.3

Table 4. Time relation in division of untreated and treated HeLa cells.

	Metaphase	Anaphase	Telophase
Control	46.2±2.02	9.2±0.65	26.2±1.39
Treated	57.0±2.53	9.0±0.93	26.3±0.93
	T= 3.198	T= 0.133	T= 0.072
	P< 0.01	P> 0.5	P> 0.5



Figs. 5–7. Showing the successive stages of mitotic inhibition in a single cell of HeLa strain after the application of Carzinophilin at a dose of 20γ per ml. Positive contrast, ×1400. 5, metaphase just before chemical treatment. 6, immediately after chemical treatment. 7, cell damaged with pycnotic degeneration of chromosomes (taken 72' after chemical treatment).

Figs. 8–10. Showing the successive stages of mitotic inhibition in a single cell of HL-C strain after the application of Thio-TEPA at a dose of 1.0 mg per ml. Positive contrast, ×1400. 8, prophase just before chemical treatment. 9, immediately after chemical treatment. 10, cell damaged with pycnotic degeneration of chromosomes (taken 34' after chemical treatment).

Mean duration of each mitotic phase of untreated and treated HeLa cells was calculated on the basis of the above data, with the results shown in Table

4 and Figure 11. In comparison with control data, it is evident that the duration of metaphase showed a striking prolongation in treated cells. Statistically the metaphase prolongation in treated cells was proved to be significant on the basis of $P < 0.01$.

3. *Effect of Thio-TEPA on dividing cells of the HL-C strain Nakanishi*

The HL-C cells were treated at metaphase with Thio-TEPA at a dose of 2.5 mg per ml. They underwent pycnotic degeneration of chromosomes after blebbing. The cells exposed at prophase or metaphase with a dose of 1.0 mg or 0.5 mg per ml were damaged through the clumping of the chromosomes (Figs. 8–10). The cells treated at prophase with 0.1 mg per ml dosage divided in an irregular manner and were damaged at metaphase in most cases, though there were a few cells which completed division. The majority of cells treated at metaphase at the same dose proceeded to telophase and then disintegrated. The cells exposed to the agent at prophase under a 0.05 mg per ml dosage completed mitotic division in regular way.

The duration of each mitotic phase ranging from metaphase to telophase was measured in the same cell under the influence of the agent at a 0.05 mg per ml dose level. The results are as shown in Table 5.

In Table 6 and Figure 12 are summarized the data dealing with the time relation in cell division of both untreated and treated HL-C cells. In reference to

Table 5. Time relation in cell division of the HL-C strain following the application of Thio-TEPA at a dose, 0.05 mg per ml.

Cell No.	Metaphase	Anaphase	Telophase
1	44min.	6min.	28min.
2	46	8	24
3	51	9	21
4	45	7	22
5	43	11	26
6	45	8	24
7	38	10	23
8	47	8	22
9	42	7	27
10	45	6	29
Average	44.6	8.0	24.6

Table 6. Time relation in division of untreated and treated HL-C cells.

	Metaphase	Anaphase	Telophase
Control	34.4 ± 1.66	8.0 ± 0.56	23.9 ± 0.91
Treated	44.6 ± 1.07	8.0 ± 0.52	24.6 ± 0.87
	$T = 4.114$	$T = 0$	$T = 0.607$
	$P < 0.01$		$P > 0.5$

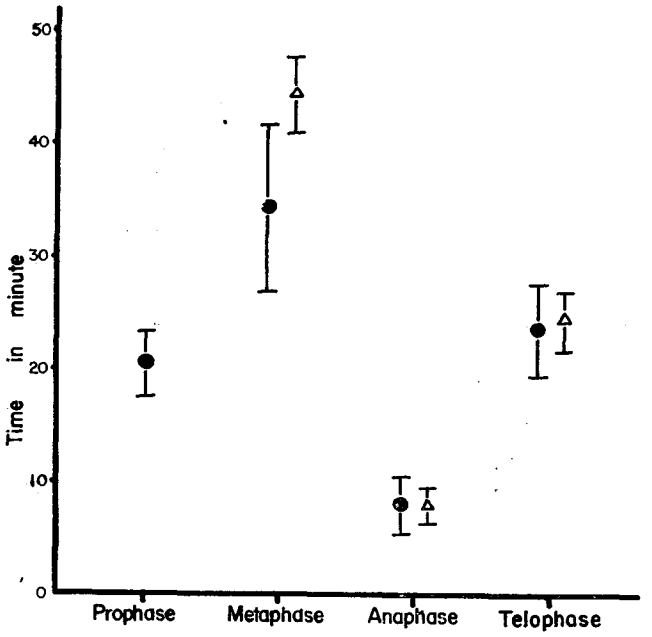


Fig. 12. Time relation in division of untreated and treated HL-C cells. At the midpoint of each phase are plotted mean durations of mitotic division (black spot, control ; triangle, treated), the standard deviation at each plotted point being indicated by a vertical bar.

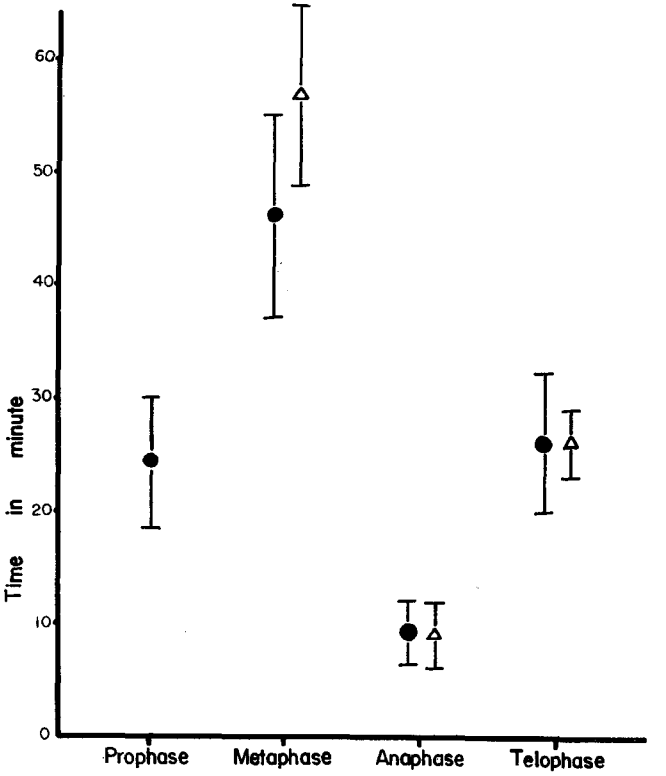


Fig. 11. Time relation in division of untreated and treated HeLa cells. At the midpoint of each phase are plotted mean durations of mitotic division (black spot, control ; triangle, treated), the standard deviation at each plotted point being indicated by a vertical bar.

the data, it is of interest to see that the duration of anaphase and telophase showed no consistent difference between untreated and treated cells, while the duration of metaphase was longer in treated cells than in untreated ones. This difference was proven to be significant statistically ($P<0.01$).

Discussion

Hori and Sasaki (1959) working with rat fibroblasts *in vitro* reported that Carizonophilin damaged primarily the nuclear chromatin and chromosomes with specific action. They observed an unusal aggregation of chromatic materials into several small masses. Awa (1958), who studied the *in vivo* effect of the same drug on the tumor cells of the MTK-sarcoma III of rats, observed the agglutination of chromosome threads into several clumps in nuclei of the treated cells. Further, Nakanishi and Makino (in press) observed by the method of phase cinematography a similar type of coagulation of chromatin substances in resting nuclei of HeLa cells in response to Carzinophilin. Working with cultured cells of mouse fibroblasts and of CBA mouse mammary adenocarcinoma, Awa (1961) found that Thio-TEPA induced severe clumping, fragmentation and translocation of metaphasic chromosomes and their sticky bridges at ana- and telophase. Evidence presented by those authors indicates that both Carzinophilin and Thio-TEPA exert specific action on the nuclear substance, and that cytoplasmic damage is less pronounced under their influence. A similar situation was found to occur in tumor cells of the MTK-sarcoma III of rats in response to Carzinophilin and Thio-TEPA (Awa 1959). The present experiments have shown that the cells treated at comparatively higher dosages of those drugs showed remarkable mitotic abnormalities, with severe clumping of the chromosomes.

Based on the results of direct observations of living *Chortophaga* neuroblasts following X-irradiation, Carlson (1941, 1950) has postulated that there exists a critical period between late and very late prophase in response to X-rays. Neuroblasts that had passed this stage at the time of treatment completed mitosis with little or no delay, when the dose was less than 250 r. According to Nakanishi (1959), a decreased order of sensitivity of grasshopper spermatocytes to beta-rays based on the degree of the stickiness of chromosomes is arranged as follows: diakinesis, metaphase and anaphase. Further there was no observable change in cell division, when irradiation was done at telophase.

Makino and Nakanishi (1955) studied the effect of podophyllin on living tumor cells of the MTK-sarcoma III of rats, and reported that metaphasic cells were damaged by a pycnotic degeneration of the chromosomes. The present experiments have shown that the cells treated at prophase with either Carzinophilin or Thio-TEPA at higher dosage failed to continue division, while the cells treated at metaphase completed division in a rather regular manner at least as observed through phase optics. The evidence leads to the inference that there occurs a critical period between prophase and metaphase in cells in response to these chemicals.

Makino and Nakahara (1953, 1955), dealing with the living tumor cells of the MTK-sarcoma I of rats, noted a relationship occurring between the variation of temperature and the length of mitotic phases, indicating that tumor cells of

similar dimension showed a remarkable decrease in the duration of each mitotic phase with rising temperature. This decrease was especially striking in metaphase. In the present study, the prolongation of the duration of metaphase was also especially remarkable in the cells treated with either Carzinophilin or Thio-TEPA. The data at hand are not sufficient for the analysis of the cause and mechanism of the prolongation of the duration of metaphase. It seems probable however that the elongation of the duration of metaphase may be a result of decreased metabolic activity as a whole in cells under the influence of chemicals, rather than the results of damaging of particular elements such as the spindle body or chromosomes. The mechanism of antimitotic action has been admirably summarized by Biesele (1961), especially from the cytochemical stand point. He has pointed out that antimetabolite action on cancer cells is often expressed in one or several antimitotic effects such as preprophasic inhibition, spindle poisoning, metaphasic delay, chromosome poisoning, and difficulties in cytokinesis.

Summary

This paper deals with a phase microscopy study on the effects of Carzinophilin and Thio-TEPA on dividing cells of the HeLa and HL-C strains.

Most cells exposed at prophase to Carzinophilin at a 5γ per ml dose level, or to Thio-TEPA at a 0.05 mg per ml dose level, completed division with a noticeable prolongation of the duration of metaphase. The application of the drugs at higher dosages at prophase or metaphase resulted in cell damage with the clumping of chromosomes. Evidence was presented to show that there existed a critical period between prophase and metaphase in cells in regard to response to chemicals.

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