The Cytological Effect of Chemicals on Tumors, IX. Cytological Response of the MTK-Sarcoma III Cells to Carzinophilin and Thio-TEPA

By

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(With 4 Text-figures and 1 Plate)

Carzinophilin is one of the antibiotics obtained from a microorganism, *Streptomyces sahachiroi*, by Hata *et al.* (1954). It has been shown that this agent exerts inhibitory effect upon tumor cells of rats and mice (Kamada *et al.* 1958, Hori and Sasaki 1958, 1959, Awa 1958).

Like nitrogen mustard and triethylene melamine (TEM), triethylene thiophosphoramide (thio-TEPA) was found to be an alkylating antitumor agent. The effectiveness of thio-TEPA upon tumors is striking (Goldie *et al.* 1957), but different in response to different experimental tumors (Sugiura and Stock 1955, Awa 1959).

Cytological studies on the effects of certain chemicals upon rat ascites tumors have been undertaken by Makino and his co-workers (Makino 1957, 1959). A major conclusion obtained by them was that, although temporary regression of the tumor growth was maintained as a result of considerable damage to most tumor cells, renewed growth of the tumor was caused by the proliferation of the tumor cells which remained alive undamaged by the drug (Makino 1957). The present study was carried out in a hope to inquire into the action of Carzinophilin and thio-TEPA on tumor cells of the rat ascites tumor, with special reference to the cytological details of the effect of these chemicals.

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Material and Methods: Rats of pure Wistar and Long-Evans strains, weighing 60 to 100 grams, were used for transmission of tumors. The ascites tumor used was the MTK-sarcoma III which malignantly propagates in the peritoneal cavities of rats. On the 3rd or 4th day after inoculation of the ascites tumor, the tumor-bearing animals received intraperitoneal injections of Carzinophilin or thio-TEPA. The tumor was sampled from treated animals at 1, 3, 6, 12, 24, 36, 48, 72 and 96 hours after injections of the agents. The preparations of tumor cells for cytological observations were made by squashing the tumor with acetic dahlia. For detailed morphological observations, Feulgen nucleal reaction and May-Grünwald Giemsa staining method were adopted.

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Observations

1) *Effect of Carzinophilin*

Carzinophilin (KYOWA) for application was prepared by dissolving the drug with a 1 per cent sodium bicarbonate solution. The solution thus prepared contains 2500 units or 25 γ of Carzinophilin in 1 cc of a 1 per cent sodium bicarbonate solution. Dilution of Carzinophilin was made by the addition of biological salt solution to an appropriate concentration. On the 3rd day transfer of the tumor, tumor-bearing rats received a single intraperitoneal injection at a dose level of 1000 units per 1 kg of body weight. Further experiments were made by the application of Carzinophilin such as 2500 and 5000 u /kg.

Generally, tumor inhibitory activity of Carzinophilin was rather slow as compared with that of other antitumor agents. Within 3 to 4 hours after the application no visible changes either in the nucleus or in the cytoplasm of the tumor cells were observable.

It was found after the first 6 to 24 hours of treatment that Carzinophilin at a dose of 1000 u /kg did attack the nucleus and the nucleolus in the treated tumor cells: striking swelling and loss of stainability were remarkable in the nucleus. The Feulgen method revealed that most affected cells showed the nuclei which stained poorly and heterogeneously. The affected nuclei were characterized in each case by a coarse network structure or irregular condensation of chromatins (Figs. 14 and 15). With the passage of time those damaged nuclei seemed no longer to undergo normal mitotic behavior.

Swelling of nucleoli in a diffused condition was particularly striking in the treated cells. In most cases they were considerably elongated and showed amoeboid or filamentous protrusions (Fig. 6). Their stainability became very irregular. In an advanced condition, small vacuoles were observable in each nucleolus.

The cytoplasm of the treated cells was also marked by the loss of stainability with dyes and by an increase in volume. Sometimes there was occurrence of cytoplasmic vacuolization specially in the peripheral region. Breaking down of the cytoplasm was finally induced. These cytoplasmic irregularities were, however, not so pronounced as compared with those observed in the nucleus or in the nucleolus. Closely associated with these cellular degenerations, a decrease in frequencies of mitotic cells took place during 24 hours after treatment (Fig. 1A). Mitotic cells which were rarely observable showed the chromosomes forming by means of association with a chromatic mass or masses.

Further marked changes in treated tumor cells were observed in the following 24 hours after the drastic application. Pronounced chromosomal abnormalities and mitotic disturbance were produced through the increase of mitotic frequency: it was of particular interest that most metaphasic chromosomes became slender and transformed into a bead-like appearance (Fig. 7). They

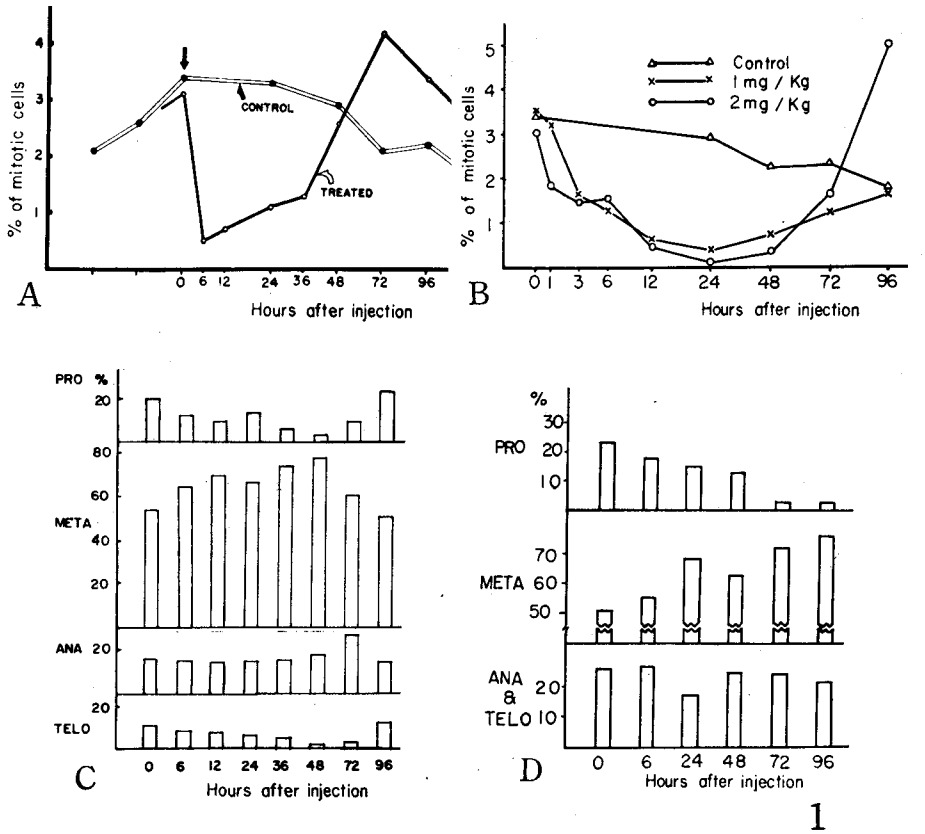


Fig. 1. A–B. Curves showing mitotic frequencies of the MTK-sarcoma III cells in treated and control animals. (A) Carzinophilin (1000 u/kg), (B) thio-TEPA (1 and 2 mg/kg). Figure 1C and D. Histograms showing relative frequencies of mitotic cells after the treatment with (C) 1000 u/kg of Carzinophilin and (D) 1 mg/kg of thio-TEPA in the MTK-sarcoma III.

were found in a number of fragments, or showing translocations of various types (Figs. 3 and 7). Bridge-formation, laggards and heavy stickiness of chromosomes were common at ana- and telophase. As seen in Figure 1C, a gradual increase in relative ratio of metaphasic cells in the tumor after the drastic application indicated the occurrence of mitotic delaying or metaphase block. With the passage of time, the drug exerted damage to tumor cells at the resting stage.

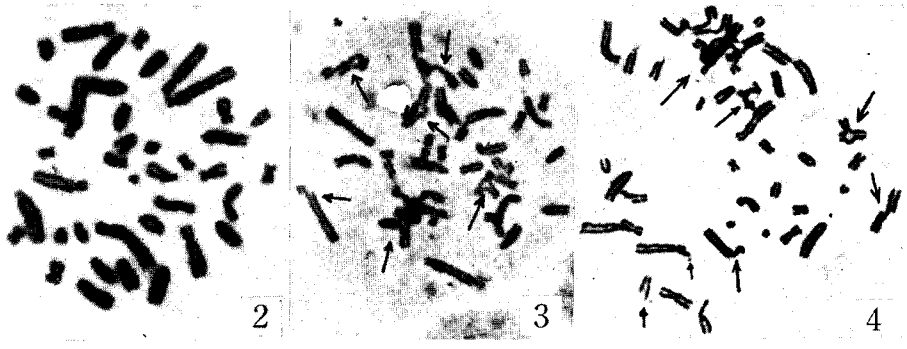
By 72 hours after treatment severe damages to most tumor cells were caused. Close observations of samples at this stage made it clear that there occurred, in the samples, tumor cells each of which was characterized by a small-

sized and a compact nucleus ; they remained alive undamaged by the drug (Fig. 8). Referring to the evidence presented by Makino and his co-workers (cf. Makino 1957, 1959), it is clear that they are residual tumor cells having been left without being broken down by the drug, and constitute a primary source of renewed growth by proliferation when the tumor becomes free from the action of the drug.

Higher dose levels such as 2500 and 5000 u /kg showed very severe damages to both the nucleus and the cytoplasm within 24 hours after treatment (Fig. 9) : metaphasic chromosomes became highly clumped.

The treatment of the tumor with Carzinophilin induced a temporary prolongation of life span of the treated animals bearing the MTK-sarcoma III. Generally, the mean life span of the treated animals showed a prolongation of about 2 or 3 days in the application of 1000 u /kg, 3 to 4 days at the dose of 2500 u /kg and more than 2 weeks in the case of 5000 u /kg, whereas the untreated animals showed a mean life span of 8.9 days.

On the basis of the above results, it may be concluded that Carzinophilin exerts influence initially upon interphase cells : such cells showed nuclei and nucleoli which were severely damaged. Then mitotic inhibition and irregularities follow.



Figs. 2 to 4. Metaphase chromosomes of the MTK-sarcoma III in treated and control cells (water pretreatment, acetic dahlia). × 1000. Fig. 2, control, 40 chromosomes. Fig. 3, Carzinophilin-treatment (1000 u/kg), 30 hours after injection. Fig. 4, thio-TEPA-treatment (1 mg/kg), 24 hours after injection. Arrows indicate translocation and fragmentation of chromosomes.

2) *Effect of thio-TEPA*

A suspension of thio-TEPA (triethylene thiophosphoramide or "Tespamin", SUMITOMO), was made with 0.5 cc of polyethylene glycol and benzyl alcohol mixture, and then the suspension was dissolved in 1.5 cc of sterilized distilled water. The solution thus prepared was diluted with biological salt solution to appropriate concentrations. Dose levels used in the present experiment were 1, 2 and 5 mg /kg

of body weight. Procedure for the treatment of the tumor-bearing rats was similar to that of Carzinophilin. For detailed cytological observations a single intraperitoneal injection at a dose level of 1 mg/kg was applied to rats bearing the MTK-sarcoma III.

During the first 24 hours after application mitotic frequencies showed a gradual reduction in all samples obtained (Fig. 1B). Morphological ly, no visible changes were observed in the tumor cells following the drastic application.

In the 24 hours next following after application, striking changes occurred in dividing tumor cells ; chromosomes in the mitotic cells underwent severe damage. As seen in Figure 4, many chromosome fragments and translocations of chromosomes were common at metaphase. They seemed to induce di- and acentric chromosomes. Additionally to these metaphasic irregularities, in some samples there occurred bridges, lagging, stickiness and distorted scattering of chromosomes at ana- and telophase. Multipolar divisions were not uncommon (Fig. 10). The cells having one or more micronuclei increased in number within 96 hours after treatment ; about 50 per cent of tumor cells observed showed micronuclei. It is of special importance to observe that the relative frequency of prophasic cells decreased, with the increase in frequency of metaphasic ones with the passage of time (Fig. 1D). The evidence presented seems to suggest that the mitotic division has been inhibited and delayed. There is a possibility that these chromosomal anomalies were induced by abnormal DNA synthesis caused by the application of thio-TEPA. No quantitative study on DNA contents was made by the author, but the following observed facts seem to support this possiblility. Response of treated nuclei to the Feulgen reaction was strikingly reduced as compared with the results in case of untreated cells : chromatin elements heterogeneously condensed and localized close to the nuclear memberane (Figs. 16 and 17). Increase in the volume of the nuclei was very remarkable. Most of the nuclei thus affected showed finger-like protrusions or were multilobated. Nucleolar traces were shown by the Feulgen positive substance which is probably heterochromatin. The outline of the nucleolus was quite irregular (Fig. 17).

The cytoplasm showed remarkable swelling within 24 hours after treatment. Small vacuoles, several in number, were found scattered in the cytoplasm.

The samples taken within 96 hours after administration showed many tumor cells in which the nuclei has undergone degeneration through karyolysis. With the passage of time, tumor cells decreased in number. There occurred many tumor cells which remained unaffected and infiltrated into the omentum. They are the residual tumor stem-cells which seem to cause the tumor regrowth by their proliferation.

Administration of the drug at higher doses resulted in severe karyolysis in tumor cells, and their rapid degeneration.

Based on the results here reported, the conclusion may reasonably be drawn that thio-TEPA attacks the tumor cell nuclei and induces chromosome breakage.

Various chromosomal abnormalities followed by mitotic disturbance may be attributed to the disturbance of nucleic acid metabolism in the affected cells.

Discussion and conclusion

Most antitumor agents such as antibiotics, steroids, alkylating agents, antimetabolites, plant extract derivatives have been known as cytotoxic agents or mitotic poisons (Makino 1957, 1959, Makino and Tanaka 1953, Stock 1954, Biesele 1958a, b). In the present study, the effects of Carzinophilin and thio-TEPA upon tumor cells were investigated by way of comparison in respect to the following points: (1) initial attack upon the nucleus in resting tumor cells, (2) induction of various chromosomal abnormalities with mitotic disturbance, and (3) less effectiveness upon the cytoplasm than upon the nucleus.

It seems probable that a striking reduction of nuclear stainability to the Feulgen reaction and irregular condensation of chromatin may be attributable to biochemical changes in DNA-content or synthesis. As a result, subsequent mitotic abnormalities or inhibition of mitosis may possibly be induced. Chromosomal abnormalities resulting from application of these two agents are highly striking in every mitotic phase.

Such abnormalities are characterized by translocation and fragmentation of chromosomes at metaphase, bridge-formation, laggards and scattering of chromosomes at ana-and telophase. Decrease in relative ratio of prophasic cells is also pronounced.

In Carzinophilin-treatment, most metaphasic chromosomes become slender until they are thread- or bead-like in appearance, whereas thio-TEPA treatment shows no such structural changes at all. Similar results have been obtained by Hori and Sasaki (1958, 1959) in the study of the effects of Carzinophilin upon normal and neoplastic cells in tissue culture.

Working on the effect of nitrogen mustard upon the Walker rat carcinoma 256, Koller and Casarini (1952) informed that various sorts of chromosomal damage were induced in treated tumor cells. Thio-TEPA used in the present study is similar to nitrogen mustard in chemical nature and acts upon tumor cells as a mitotic poison. Micronuculei formation is remarkable in the affected cells. It is of interest that thio-TEPA at low concentrations exerts damage upon both the nucleus and the cytoplasm of normal as well as neoplastic cells *in vitro*, while at higher concentrations this drug induces severe nuclear degeneration in tumor cells (Awa, unpublished).

The action of Carzinophilin is remarkable by the changes occurring in the nucleolus. The nucleolar changes are characterized by irregular and filamentous protrusions like pseudopodia. In an advanced condition of cellular damage, granular vacuoles are produced in the nucleoli, and finally disintegration of nucleoli occurred. Stainability with dyes is quite irregular: sometimes it is poor, sometimes strong. Similar abnormalities have been induced by some other antibiotics such as Sarkomycin and Mitomycin (Hori and Sasaki 1958, 1959, Kobayashi 1959). It is probable that such nucleolar abnormalities occur in association with the changes in nucleolar RNA. No such remarkable nucleolar

degeneration was induced by the application of thio-TEPA.

Both Carzinophilin and thio-TEPA produce cytoplasmic damage shown by the formation of vacuoles or cytolytic disintegration, though the irregularities are less pronounced than those found in the nucleus.

The severity of response becomes increasing with increased concentrations in both Carzinophilin and thio-TEPA. The same situation was found by Makino and Cornman (1953) who investigated the effect of podophyllotoxin on malignant cells in tissue culture.

It has been shown that following the applications of Carzinophilin and thio-TEPA certain stem-cells of residual type have remained undamaged in the peritoneal cavities of treated animals, although most tumor cells were damaged by the agents used. It is apparent that these residual tumor cells form the primary source of renewed tumor growth through their proliferation (cf. Makino 1957, 1959).

Summary

The present article deals with the cytological response of tumor cells of the MTK-sarcoma III to Carzinophilin and thio-TEPA, with special regard in some detail to the course of cell damage by the agents.

Both Carzinophilin and thio-TEPA are remarkable in inducing nuclear irregularities which are characterized by heterogeneous condensation of chromatic materials and nuclear decomposition, together with various chromosomal breakages. Noticeable is the fact that Carzinophilin produces damage to the nucleolus in the treated tumor cells at any concentrations so far examined: morphologically, the nucleoli thus affected show irregular protrusions and their stainability becomes abnormal as compared with those of untreated cells. It may explicable by the changes of DNA and RNA occurring in the nucleus.

The general inhibitory effect of these agents on mitosis of tumor cells may probably be attributable to the interference of nucleic acid metabolism in tumor cells.

There occur certain stem-cells of residual type which have remained undamaged by these chemicals: they seem to constitute the primary source of renewed tumor growth.

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Explanation of Plate IV

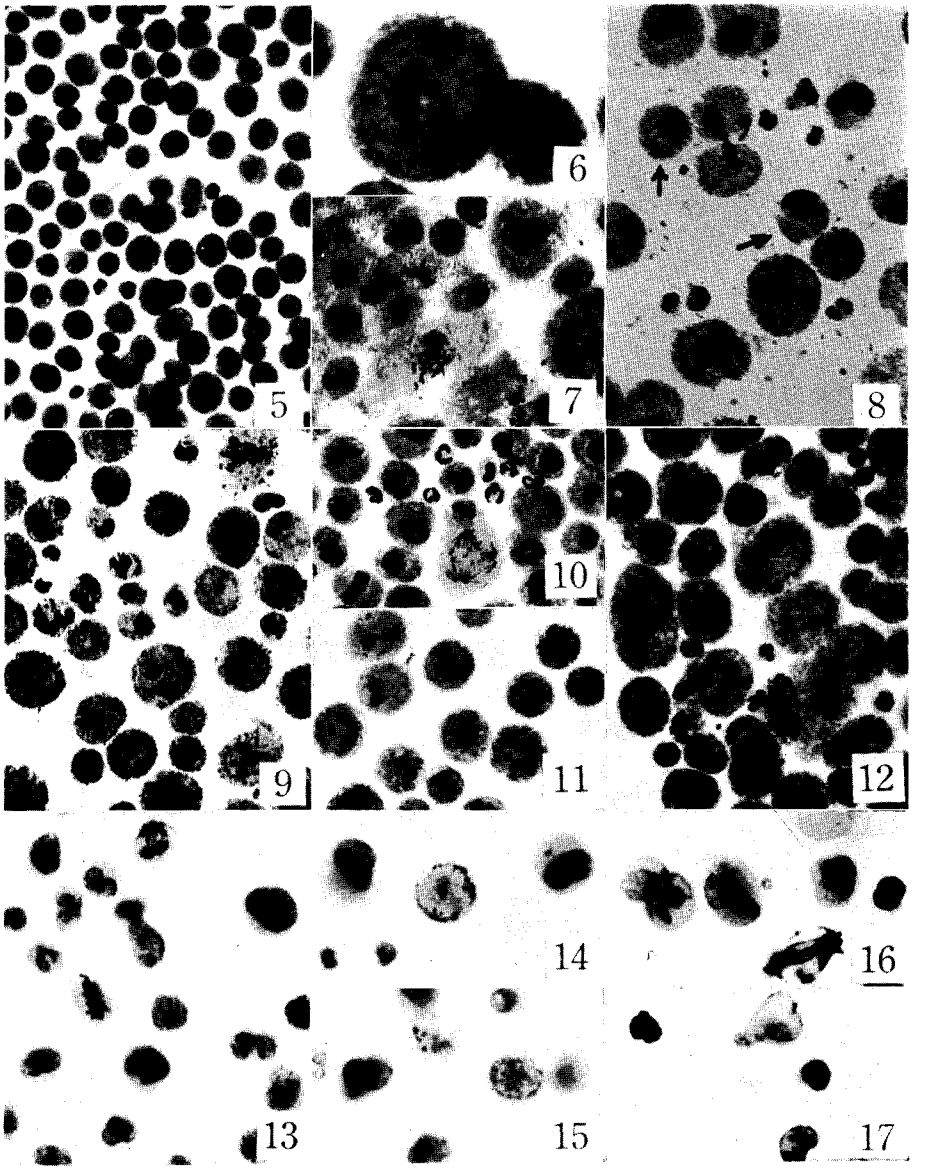
Fig. 5. MTK-sarcoma III cells of the 4th day after transplantation, just before injection (control). ×300.

Figs. 6 to 8. Destructive changes of tumor cells following Carzinophilin-treatment (1000 u/kg). Fig. 6. Elongation and thread-like protrusions occurring in the nucleolus at the resting stage, 6 hours after injection. ×1000. Fig. 7. Nuclear and cytoplasmic destruction and chromosome breakages in tumor cells, 24 hours after injection. ×400. Fig. 8. Residual cells (indicated by arrows) showing normal appearance, 48 hours after injection. ×400.

Fig. 9. Tumor cells in the course of damage (5000 u/kg). Note swelling of nucleoli as well as irregular outlines of chromosomes and cytoplasm. ×400.

Figs. 10–12. Degeneration of tumor cells following thio-TEPA-treatment (1 mg/kg). ×300. Fig. 10. Swelling of tumor cells and tripolar division, 24 hours after injection. Fig. 11. Striking reduction in stainability of the nucleus, together with abnormal anaphase, 48 hours after injection. Fig. 12. Irregular mitoses, together with swelling of cytoplasm and nucleus, 72 hours after injection. All are derived from smear preparations stained with acetic dahlia.

Figs. 13 to 17. Photomicrographs of tumor cells of the MTK- sarcoma III, from preparations stained with Feulgen reaction. ×750. Fig. 13. Control. Figs. 14 and 15. Carzinophilin-treatment (100 u/kg). Note irregular condensation of chromatins and nucleolus, 24 hours after injection. Figs. 16 and 17. Thio-TEPA-treatment (1 mg/kg). Striking reduction of stainability, together with abnormal anaphase, 24 hours after injection.



A. Awa: *Response of MTK-Sarcoma Cells to Carzinophilin and Thio-TEPA*