The Cytological Effect of Chemicals on Tumors, XVIII Effects of Anti-Tumor Agents on the Ameboid Motility of Tumor Cells in an Ascites Sarcoma of the Rat[1)2)]

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(*With 22 Text-figures*)

In the light of reported evidence accessible in the literature, it is generally understood that the mechanism of metastasis of tumor is closely associated with the phenomenon of tumor invasion. Coman and his coworkers (Coman 1953, Enterline and Coman 1950) studied extensively the related subject and reached the conclusion that decrease of mutual adhesiveness caused tumor cells to separate from one another and facilitated their invasion into interstitial spaces of adjacent tissues by means of their ameboid movement.

Working with ascites tumors of the rat, Nakahara (1955) and Kato and Makino (1962) reported that tumor cells were actively motile under certain conditions. Further, Hirono (1958) found a correlation between the intensity of ameboid motility and the incidence of metastatic spread in several strains of ascites hepatomas of rats. Evidence presented seems to suggest that ameboid movement appears to be a necessary prelude to the invasive activity of tumor cells.

It is, accordingly, of fundamental importance to inquire into the very nature of the ameboid motility of tumor cells, for understanding of the mechanism of tumor metastasis. The present report deals with a study on effects of two anti-tumor agents, Carzinophilin and 8-azaguanine, on the ameboid motility of the MTK-sarcoma III, an ascites tumor of the rat, with special regard to the maneuvers of tumor stem-cells in relation to motility.

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Material and Methods: The MTK-sarcoma III, a rat ascites tumor, offered the material for the present study. The tumor has since long been maintained by serial intraperitoneal transfers in rats of Wistar and Long Evans strains, weighing 80 to 110g.

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Transplantation of the ascites tumor was performed with the use of sterile fine-tipped glass pipettes with an inoculum dose of 0.05 cc.

The chemicals employed were Carzinophilin and 8-azaguanine. Carzinophilin is an antibiotic extracted from *Streptomyces sahachiroi* by Hata *et al.* (1954), and 8-azaguanine is one of the antimetabolites. They are known as anti-tumor agents. Tumor-bearing animals received a single intraperitoneal injection of the drug on the 5th day following the transfer of tumor. Dose levels for examination were 7500, 5000, 2500 and 500 units per kg of the body weight in the case of Carzinophilin, while they were 80 and 40 mg per kg in 8-azaguanine. Tumor cells were sampled from treated animals at definite intervals and observed by means of phase microscopy in preparations made through the hanging-drop method (Makino and Nakahara 1953). At the same time additional samples were squashed with acetic dahlia for cytological examinations.

Results

I. Controls

1) *Frequency of occurrence of ameboid cells after preparation*: Preliminarily the frequency of handmirror-shaped cells after preparation was studied. Usually, immediately after preparation, a certain number of cells showed active movement. However, they became spherical within fifteen minutes. Thirty to forty minutes later, they appeared again in handmirror-shape with active locomotion. In additon some cells which were spherical at the time of preparation became handmirror in shape with the lapse of time. Usually, 40 to 60 minutes after preparation the occurrence of the handmirror-cells became constant.

The results of observations noted above lead to the conclusion that the frequency of transformed cells should be examined at least 1 hour after preparation.

2) *On the frequency of ameboid cells in a transfer generation*: Seven tumor-bearing rats, which were inoculated each with 0.05 cc of 5-day-old tumor ascites, supplied daily materials throughout the whole life span for observations of the frequency of motile cells. The results are graphed in Figure 1.

Referring to the information obtained in the preceding observations, the frequency of ameboid cells was observed about 1.5 hours after preparation on the basis of 300 to 500 cells. The results gained were qualitatively almost the same in all cases: as shown in Figure 1, the ameboid cells were high in percentage on the first two days of a transfer generation, showing a peak at 18.4 per cent on the second day. Then a sudden decrease of frequency occurred on the 3rd day. This trend remained unaltered till the end of the life span, where a slight rise in the frequency was observed, being 1.9 per cent on the 6th day, whereas it was 2.9 per cent on the 8th. In Figures 2 and 3 are shown pictures of material derived from 2-day- and 5-day-old tumors, and photographed 1.5 hours after preparation, respectively.

3) *Mitotic frequency*: The daily frequency of dividing cells including those at prophase, metaphase, anaphase and telophase was observed based on 2000 tumor cells per day throughout the life span in five animals which had received 0.05 cc of 5-day-old tumor ascites. The results of counts indicated that the daily

mitotic frequency of tumor cells followed a similar pattern in those five cases; average values of the mitotic incidence are shown in Figure 1. It was shown that the mitotic index was maximum in value being 4.1 per cent on the 3rd day of a transfer generation, after which it fell gradually to become 1.4 per cent on the 8th day. It is of interest that the peak of the mitotic index followed that of the frequency of transformed cells.

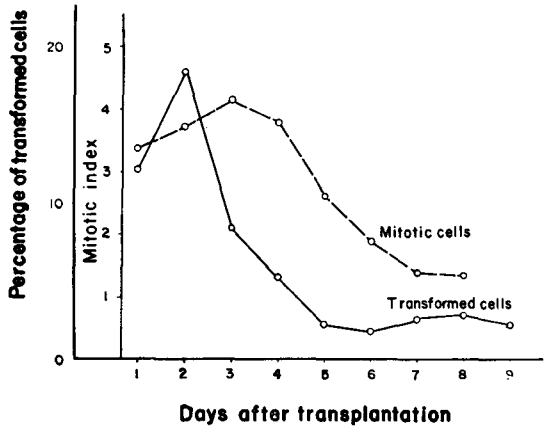
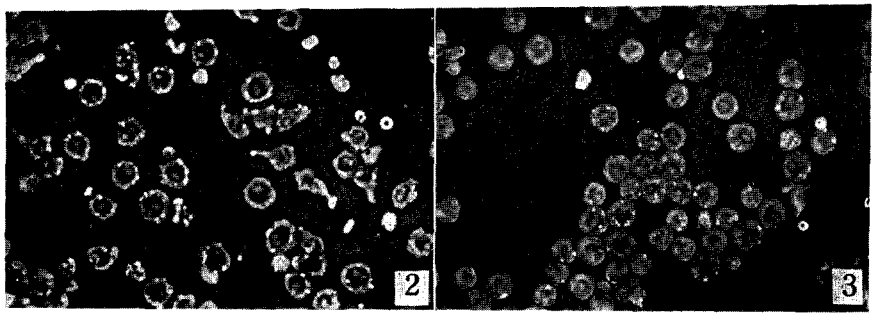


Fig. 1. Showing frequencies of transformed ameboid and mitotic cells in a transfer generation.



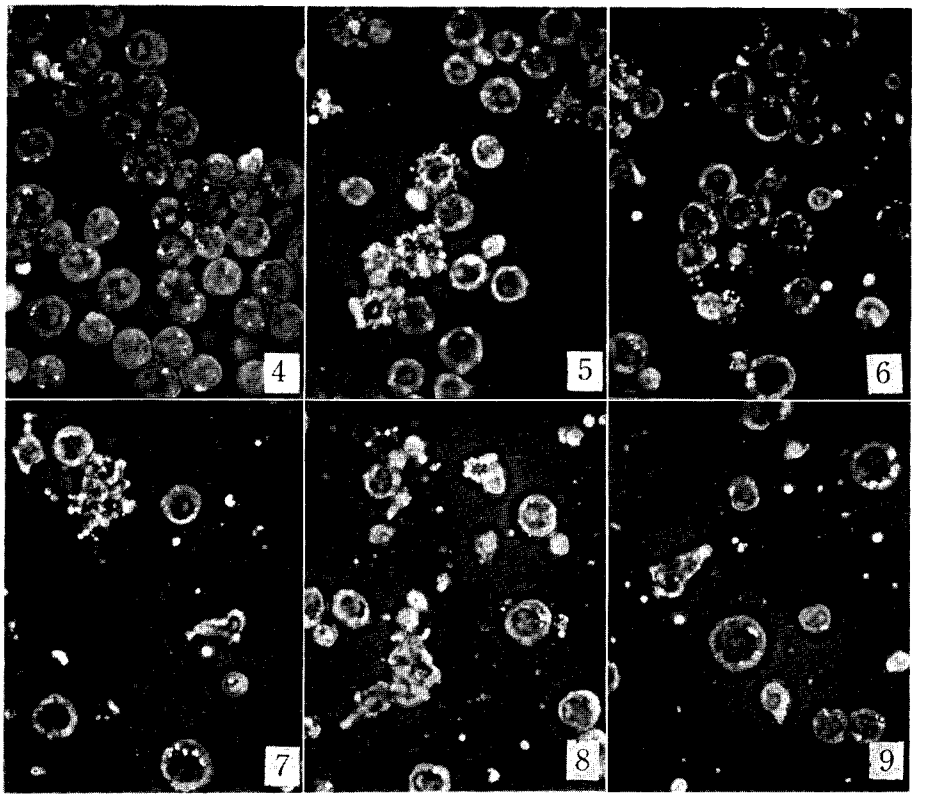
Figs. 2 and 3. Difference in frequency of transformed ameboid cells, observed on different days of a transfer generation. 2, on the 2nd day following transplantation. 3, on the 5th day. ×160, negative contrast.

II. Effects of anti-tumor agents on the frequency of ameboid cells

1) *The effect of Carzinophilin*: On the 5th day after transfer of the tumor, tumor-bearing rats received a single intraperitoneal injection of 1 ml of Carzinophilin of the following different doses: 7500, 5000, 2500 and 500 units per

kg of body weight. Cytological effects of this drug on tumor cells, especially on the nucleus, have already been reported by several workers (cf. Awa 1959), so that the present observations will be confined mainly to its influence on the ameboid motility of tumor cells.

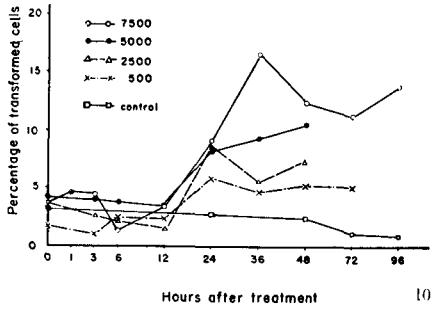
Figures 4 to 9 show successive changes in shape of tumor cells after treatment with this drug. Within 3 to 4 hours after injection of the drug no detectable change took place in the percentage of ameboid cells nor in their morphological features.



Figs. 4–9. Photomicrographs showing the effect of Carzinophilin (7500u/kg), ×320, negative contrast. 4, before treatment. 5, 6 hours later. 6, 12 hours later. 7, 36 hours later. 8, 48 hours later. 9, 72 hours later.

Severe damage to most cells was observed 6 to 24 hours after treatment. Along with this change the transformed ameboid cells greatly decreased in number. It was at these stages that the percentage of ameboid cells became minimum in value. It is remarkable however that, despite the drastic damage, there still existed several

tumor cells which remained undamaged by the drug. Furthermore, some of them assumed a remarkable handmirror shape and moved about actively in ameboid manner. During the next 24 hours, most residual cells increased in volume to a diameter 1.5 to 2 times as large as untreated cells, and a striking increase occurred in frequency of the ameboid cells (Figs. 10–11). Thirty six hours after application of the drug at 7500 units, the frequency of the ameboid cells showed 16 per cent or more, which nearly corresponds to the maximum value observed in the untreated tumor during a transfer generation. Under the continued action of the drug, a number of damaged cells were found intermingled with residual cells in samples derived 36 hours or more after treatment. Noticeable, however, was the fact that the transformed ameboid cells were different in general appearance from damaged



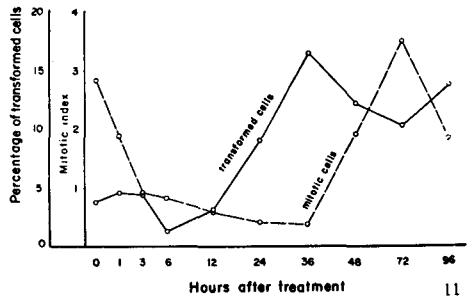


Fig. 10. Fig. 11.

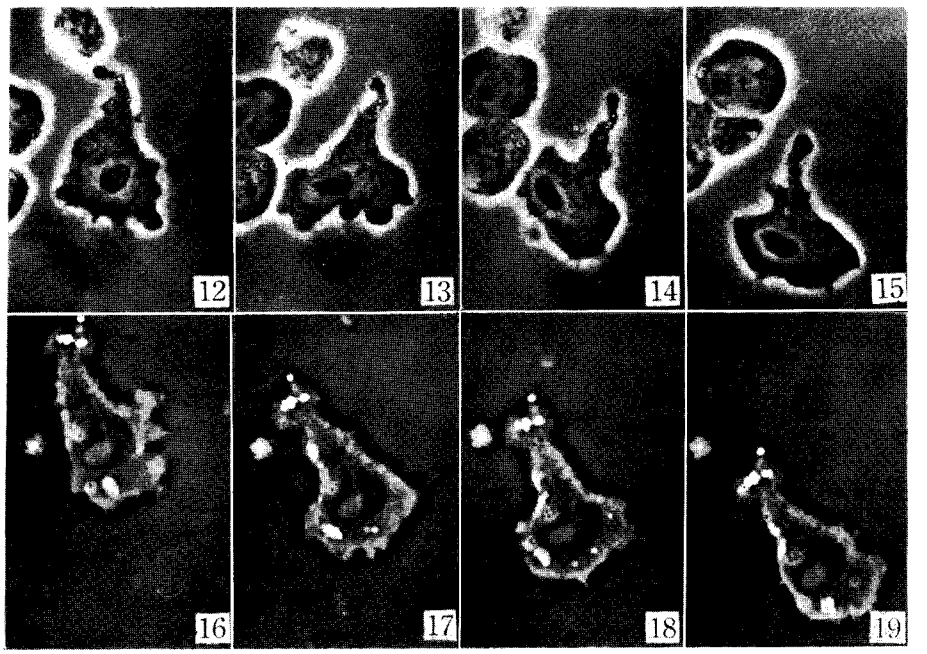
Fig. 10. A graphical representation of the frequency of transformed cells after the application of Carzinophilin at different dose levels.

Fig. 11. Shifts in frequency of ameboid and mitotic cells after Carzinophilin treatment at 7500 u/kg.

cells. Furthermore the former showed an active ameboid movement without any sign of degeneration (Figs. 12–19). The frequency tended to decrease slightly by 48 hours after injection and from then on. By 72 hours after treatment, the number of tumor cells in the ascites had decreased to a great extent. Damaged cells were no longer observable. With the passage of time, cells of regular appearance increased in number. In the ascites there were cells of regular appearance and those with vacuoles. Cells of both types appeared in handmirror shape below a certain proportion.

In an attempt to investigate the regrowth of tumor cells after severe damage caused by Carzinophilin in relation to the occurrence of motile cells, the frequency of mitotic cells was examined in samples derived from two animals treated with Carzinophilin at 7500 units. The results of the examination are shown in Figure 11 in company with those of the frequency of the ameboid cells.

On the 5th day of a transfer generation, i.e. 0 hour after treatment, the mitotic index was found to be 2.85 per cent in an average of the two animals here examined. However, within 3 hours following treatment, that index rapidly fell to 0.9 per cent, then showed a decrease until 36 hours after treatment. Thus it became 0.35 per cent. A maximum value appeared 72 hours after treatment, then it showed a considerable decrease towards the end of the life span of the host. Here it should be mentioned that the period of active proliferation of the tumor begins shortly after the ameboid motility of the tumor reaches to its peak, since a similar situation was observed also in untreated tumors.



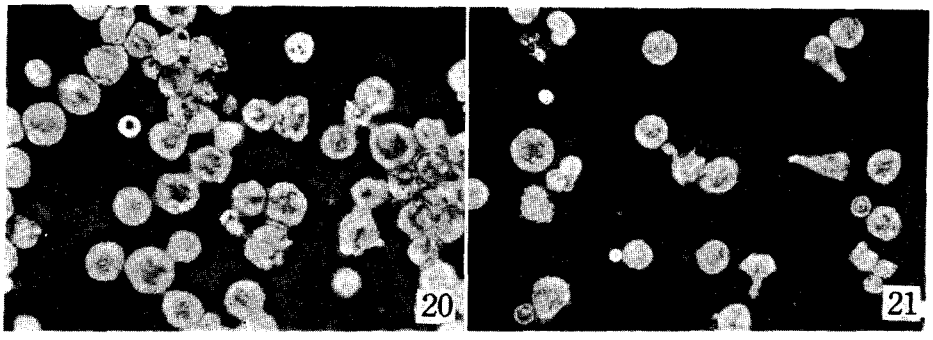
Figs. 12–19. Ameboid locomotion as compared between a chemically treated cell and a control (untreated) cell, ×600. 11.–14, control cell, positive contrast. 15–18, treated cell, 36 hours after treatment with Carzinophilin (7500 units/kg). The treated cell contains many vacuoles in the cytoplasm.

2) *The effect of 8-azaguanine*: On the 5th day after transplantation of the tumor, tumor-bearing rats received single injections of 8-azaguanine at doses of 80 mg/kg and 40 mg/kg, respectively.

Within 12 hours after application, morphologically no changes were visible in tumor cells, with an exception of a slight increase in number of vacuolized cells. In the next 12 hours, a considerable number of cells became severely damaged. As

observed in the case of Carzinophilin treatment, however, a certain number of cells remained unaffected, and moved actively in ameboid manner (Figs. 20 and 21). As shown in Figure 22, the frequency of those ameboid cells was maximum in value showing 12.7 per cent. The ameboid cells appeared somewhat earlier in samples of 40 mg/kg treatment than in those treated with 80 mg/kg.

Mitotic frequency after the application of this drug was studied in three tumor-bearing animals treated with 8–azaguanine at 80 mg/kg. Compared with the case of Carzinophilin treatment, the change of mitotic frequency was not so



Figs. 20–21. MTK-sarcoma III treated with 8-azaguanine at 80 mg/kg; ×320, negative contrast. 20, 12 hours after injection. 21, 24 hours after injection.

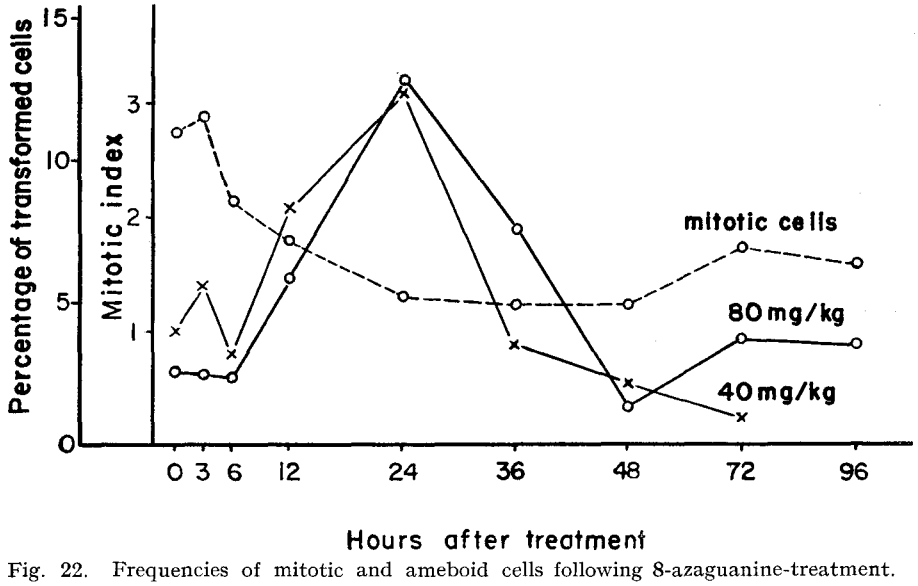


Fig. 22. Frequencies of mitotic and ameboid cells following 8-azaguanine-treatment.

drastic here: the frequency showed a trend to decrease till 36 hours after treatment, then gradually increased (Figs. 22). A tendency of the locomotive phase of tumor cells occurred also preceding their proliferation phase.

Discussion

Working on the mobility of tumor cells of rat ascites sarcomas, Nakahara (1955) informed that the tumor cells change their shape and move about only in case they infiltrated into the tissue. The present observations, however, revealed that certain tumor cells moved in ameboid manner immediately after preparation. Those ameboid cells became spherical within fifteen minutes, but they appeared again in ameboid form when the preparation was placed in a warm box. It is therefore presumable that those cells may be capable of taking ameboid form even in the peritoneal cavity of the host, to a greater or less degree. As cells require a solid substratum for their ameboid locomotion (Weiss 1961), the ameboid cells may lie in the ascites along the inner surface of the peritoneal cavity.

Recently Hori (1956) made a cytological study of the process of metastasis of tumor in the omentum of rats which had received intraperitoneal inoculation of the MTK-sarcoma III. He reported that such metastatic invasion took place by an ameboid movement of tumor cells on the mesothelium of the omentum. After invasion thereinto, the tumor cells increased in number through repeated multiplication accompanied by the resultant thickening of the texture of the omentum. The present study has revealed that through the life span of untreated tumor-bearing animals the value of mitotic index of the tumor does not go parallel to the frequency value of ameboid cells (see Fig. 1). A similar trend was found to occur in chemically treated tumors. It is a general feature that following the treatment of tumor with either Carzinophilin or 8–azaguanine, the regression of tumor always took place through a large number of tumor cells underwent damage. However, there was a certain number of tumor cells which remained alive unaffected by the drug. Some of those residual cells showed active mobility. The mitotic rate showed the peak shortly later (Figs. 11, 22). The situation observed here is very similar to that noted by Hori (1956) in the study of metastasis. Lubinska (1961) observed with time lapse films that Schwann cells cultivated *in vitro* first migrated and then dispersed in disorderly manner from an explant. Mitoses usually occurred in those dispersed cells. Since the active proliferation of tumor cells always follows their locomotive phase, it is inferred that an intimate correlation may exist between ameboid locomotion and a certain kind of cellular activity. On the other hand, ameboid motility seems to play a role in infiltration of the tumor. Taking the above features into consideration, it seems very likely that the ameboid movement of tumor cells is not an indication of degenerative process at all.

Makino and his coworkers (cf. Makino 1957) have undertaken an extensive study on the cytological effects of anti-tumor agents upon tumor growth. The general conclusion has been drawn that temporary regression of tumor growth was induced as a result of damage to a large number of the tumor cells, some of which

remained unaffected by the action of drugs and furnished the primary source for renewed malignant growth. Those residual cells were referred to as stem-cells which are principal contributors to the renewed growth of tumor. It was shown in the present study that the residual cells moved actively with ameboid activity. Therefore it seems very likely that those ameboid cells are no other than the tumor stemcells, though not all. Furthermore, it was found that the mitochondria occurring in those ameboid cells were mostly filamentous in outline. Working on the mitochondria in the MTK-sarcoma III, Okada and Nakahara (1956) stated that tumor cells having filamentous mitochondria seemed to correspond to the stem-cells at the resting stage. There exists a possible correlation between tumor stem-cells and ameboid cells in the light of the above evidence.

Summary

Ameboid motility was studied in tumor cells of the MTK-sarcoma III through the life span of tumor-bearing rats under non-treated and chemically treated conditions. It was found that under non-treated condition the phase of ameboid motility in tumor cells was not parallel to or over that of the proliferation of the tumor. Similarly, an active regrowth of tumors following drastic damage by Carzinophilin and 8-azaguanine was always preceded by the phase of striking ameboid motility of unaffected residual cells.

In the light of the findings, possible conclusions were drawn that the ameboid movement of tumor cells may be intimately associated with a certain cellular activity at the time of the growth of tumor, and that residual cells showing ameboid movement after chemical treatments may correspond to tumor stem-cells, primary contributors to tumor growth.

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