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**Studies on the Cytoplasmic Granules in the Tumor
Cells of the MTK-sarcoma, I. Morphological
Observations on the Neutral Red Granules¹⁾**

By

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(With 11 Figures)

It has been shown by Shear and Belkin (1937), Bourine (1942), Ludford (1942), Dalton & Earle (1944), Zollinger (1948), and Dalton et al. (1949) that in both normal and malignant cells the morphological changes of the cytoplasmic granules have close relations with the physiological function of cells. The mutual relation between the morphological features of the cytoplasmic granules in cells, especially in tumor cells exerting active mitotic manoeuver, and their physiological functions is very important.

Recently two different kinds of transplantable ascites tumors in Wistar white rats were established in our laboratory through the efforts of Dr. T. Tanaka. They were induced by application of azo dyes and called the MTK-sarcoma I and II, respectively (Tanaka and Kanô 1951). Both tumors proved to be favorable material for cytological investigation because of their several profitable advantages. In the present study the author investigated the morphological features of the cytoplasmic granules occurring in tumor cells of the MTK-sarcoma I and II, with particular interest in the relation between the change of the cytoplasmic granules and the mitotic activity of tumor cells.

The author wishes to express his sincere gratitude to Professor Sajiro Makino, under whose guidance the work has been continued, for his frequent valuable suggestions and for the improvement of this manuscript for publication. To Mr. T. Tanaka, Mrs. K. Kanô and Mr. H. Nakahara, the author is also greatly indebted for their kind aid in various ways.

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Jour. Fac. Sci., Hokkaido Univ., Ser. VI, Zool., 12, 1954.

Material and methods

The Wistar albinos (*Rattus norvegicus*) purely bred in this colony were used for transmission of the tumor.

Morphological observations on the neutral red granules occurring in tumor cells of the MTK-sarcoma I and II, mainly the MTK-sarcoma II, were carried out with daily material through the whole life span of certain tumor rats. The supravital staining method with the application of neutral red was tried for the preliminary observations. But this method was proved unsuitable because of being injurious to cells after immersion for a long time. Accordingly the present observations were made with the application of the new method devised by Yoshida (1949). The procedure is as follows: A drop of neutral red solution, which is dissolved in salt solution at 0.02 %, is mixed with a drop of ascites and then the mixture is covered with a cover slip.

In order to observe the intraperitoneal cellular reaction, especially of tumor cells, induced with chemicals, 1 cc of 25 mg α -peltatin solution dissolved in distilled water was injected into the peritoneal cavity of a tumor-bearing rat on the 4th day after transplantation. At constant intervals after treatment, the ascites was taken out from the treated animals and then observed, as outlined above, by means of supravital staining with neutral red.

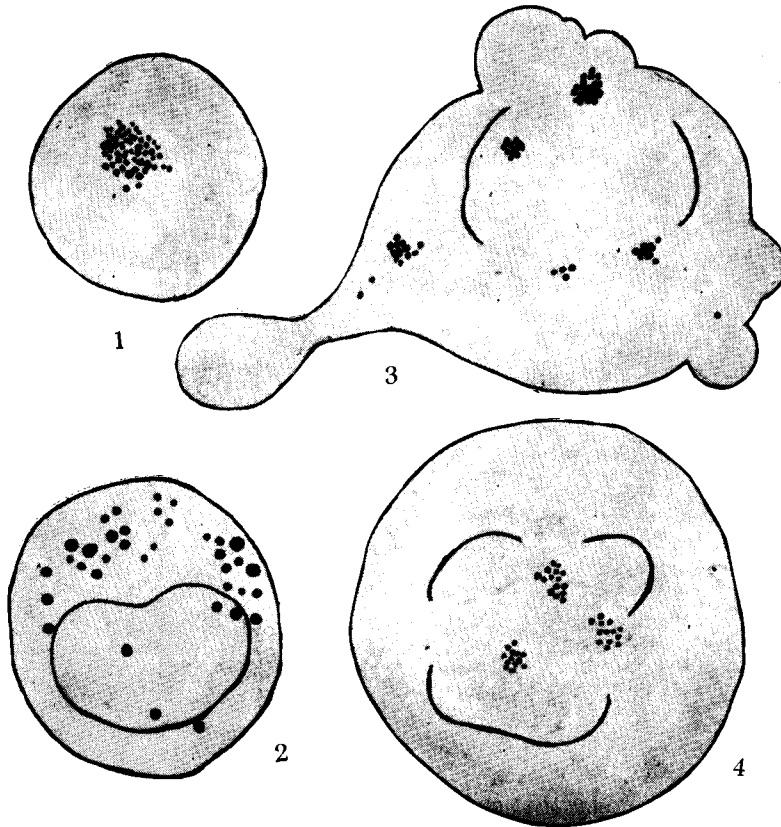
Results

1) *Granules demonstrated by neutral red*: The application of supravital staining method with neutral red demonstrated the granules stained reddish in the cytoplasm. Generally the granules were not similar in morphological features. With regard to the morphological features of the granules, the tumor cells of the MTK-sarcoma II were classified into two types as follows:

I) R_1 -type; the cells of this type contain the neutral red granules which arrange in a rosette form in the cytoplasm (Figs. 1 and 5). Granules scattered near the nucleus were comparatively smaller than those distributed in the peripheral part of the cytoplasm. Sometimes, the granules colored light-green could be seen scattered in the peripheral part of the cytoplasm. They were variable in number and observable either at resting stage or at prophase. At prophase, especially early prophase, neutral red granules showed a typical rosette form, while at later prophase they did not take a rosette arrangement. At metaphase, neutral red granules disappeared but those stained light-green have remained without change through metaphase, anaphase and telophase. After the reconstruction of daughter nuclei again at telophase, neutral red granules made their appearance being arranged in a rosette form in the cytoplasm. The evidence presented above seems to show that neutral red granules and those of light-green color must be independent in their nature.

II) R_2 -type; the cells of this type are characterized by the appearance

of vacuoles of varying sizes stained with neutral red, together with those stained reddish or light-green irregularly distributed in the cytoplasm. It seems that the cells of the R_2 -type are those in process of degeneration (Figs. 2 and 6).

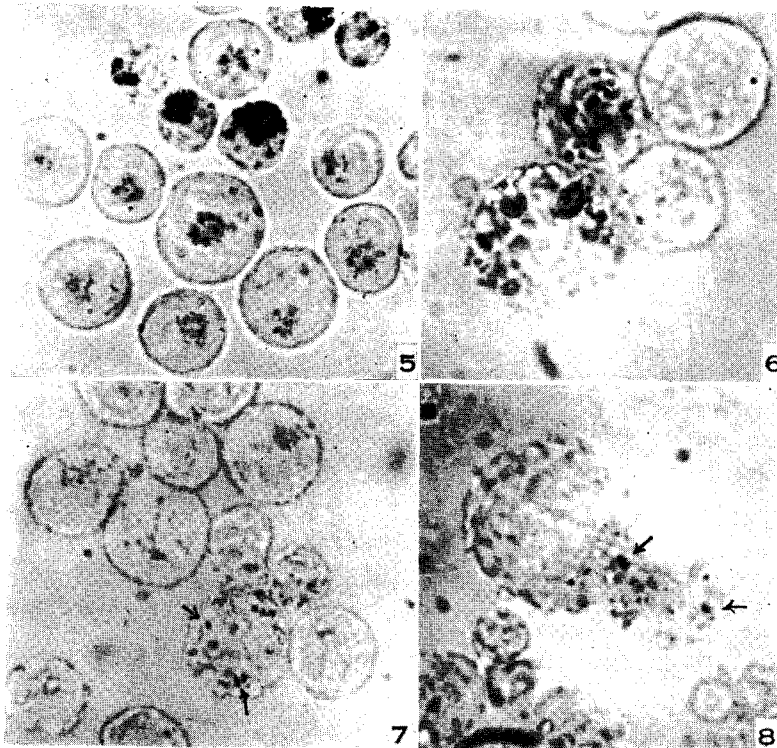


Figs. 1-4, camera-lucida drawings of tumor cells, *ca.* $\times 3200$. Fig. 1, tumor cells with neutral red granules in a rosette form (R_1 -type). Fig. 2, tumor cells with neutral red granules and vacuoles (R_2 -type). Figs. 3-4, showing agglutination of neutral red granules induced by α -peltatin treatment.

Throughout a transplant generation of the MTK-sarcoma II, the daily frequency of the cells of both R_1 - and R_2 -types was observed. On the 3rd and 4th days after transplantation of the tumor, the cells of R_1 -type showed a high frequency in appearance, while those of R_2 -type were greater in number than those of R_1 -type on the 1st and 7th day.

2) *Observations on the granules stained by neutral red in a transplant generation of the MTK-sarcoma II:* Through a transplant generation of the

MTK-sarcoma II, the daily frequencies of the cells with neutral red granules, and those of the R_1 - and R_2 -types were studied by way of comparison. The life span of the tumor-bearing rat for this study was 9 days. For comparison the daily frequency of the mitotic tumor cells was observed with the smear preparations sampled from the same tumor and stained with acetic dahlia. The data are outlined in Fig. 9.



Figs. 5-8, photomicrographs of tumor cells. *ca.* $\times 1000$. Fig. 5, tumor cells with neutral red granules arranged in a rosette form (R_1 -type). Fig. 6, tumor cells with neutral red granules and vacuoles (R_2 -type). Figs. 7-8, showing agglutination and dispersion of neutral red granules induced with α -peltatin treatment (indicated by arrows).

On the 1st day after transplantation of the tumor, the cells of the R_2 -type showed a high frequency in appearance, while those of the R_1 -type were few in number. On the 3rd day the cells of the R_2 -type gradually decreased in number, and were replaced by cells of the R_1 -type which showed a gradual increase in frequency towards the middle part of the life span of the tumor-bearing rat. On

the 4th or 5th day, R₁-type cells showed the highest frequency in appearance. Toward the latter part of a transplant generation, the cells of R₂-type showed again a gradual increase. On the 8th day, the day preceding the death of the host, the cells of R₁-type showed remarkable decrease comprising only 10 percent of the observed cells, with the considerable increase in frequency of the cells of R₂-type showing about 70 percent. On the other hand, the mitotic cells with regular behavior were the highest in frequency on the 3rd or 4th day after transplantation. Thereafter, they showed gradual decrease towards the latter part of the transplant generation.

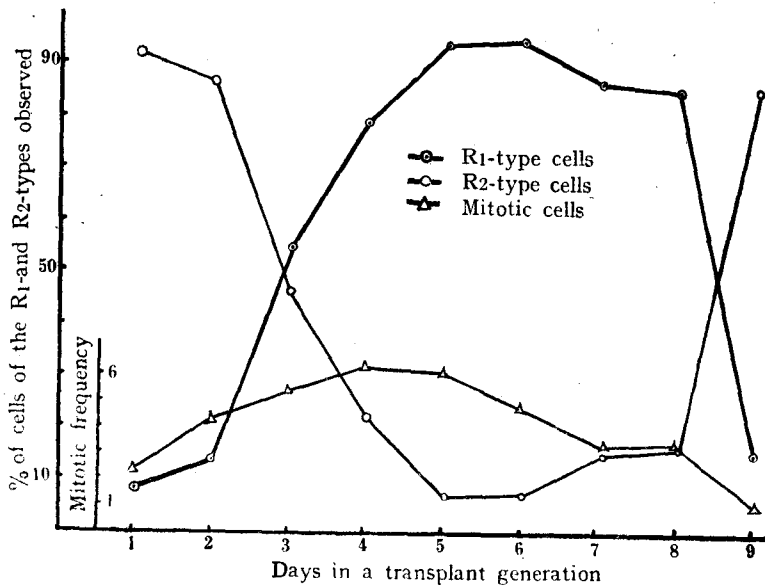


Fig. 9. Graphical representation showing the daily frequency of the tumor cells of R₁- and R₂-types through a transplant generation of the MTK-sarcoma II.

The foregoing observations indicated a somewhat parallel occurrence of the R₁-type cells and mitotic cells; that is, they showed an increase in frequency from the early part of the transplant generation through the middle part, and decreased towards the end of the generation. Makino (1952), and Makino and Kanó (1951, 1953) have given sufficient evidence to show that the mitotic cells undergoing regular division are stemline-cells which primarily contribute to the growth of the tumor. The evidence presented here seems to imply that the cells of the R₁-type are to be regarded as the stemline-cells in a resting condition.

3) *Feature of granules in tumor cells after treatment with α -pellatin*: From

the above evidence it was suggested that morphological features of the cytoplasmic granules of tumor cells seem to have a parallel relation to their metabolic activity, especially in stemline-cells. The following experiments were undertaken to ascertain this relationship. The chemical used here was α -peltatin.¹⁾ This drug is a crystalline compound isolated from podophyllin and known to be a powerful mitotic poison (Leiter, Downing, Hartwell and Shear 1950). The observations were carried out with special regard to the change or changes of granules of tumor cells induced through the application of α -peltatin. Intraperitoneal injections of 1cc of α -peltatin were made in the tumor-bearing animals on the 4th day after transplantation, and the morphological changes of the cytoplasmic granules of tumor cells were investigated using the supravitral method till 48 hours after injection. The data obtained are outlined in Figures 10 and 11.

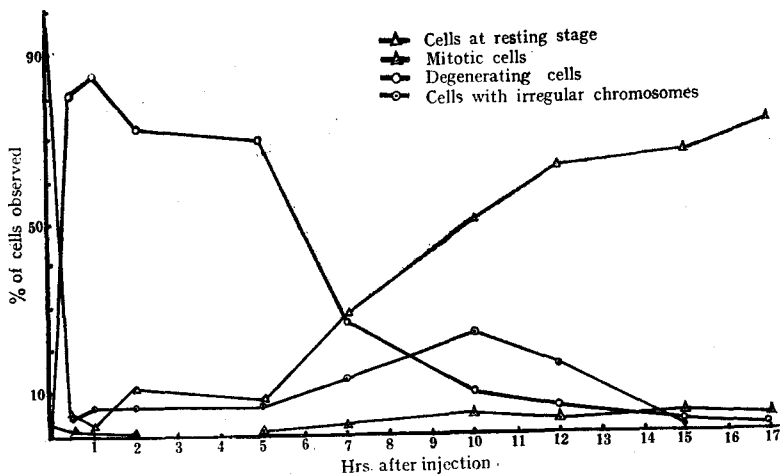


Fig. 10. Graphical representation showing damage of tumor cells induced with α -peltatin treatment in the MTK-sarcoma II (acetic dahlia preparations).

Before treatment with the drug, the tumor ascites showed that most of the tumor cells were R_1 -type, those of the R_2 -type being very rare in occurrence. About 30 minutes after injection, the R_2 -type cells made their appearance, great in number, together with many tumor cells characterised by cytoplasm stained red. The latter were probably damaged by the chemical, and showed many granules which were found diffusely scattered or agglutinated in the cytoplasm (Figs. 3-4 and 7-8). Generally, non-treated dividing tumor cells showed no neutral

1) The drug used here was supplied by Dr. M. J. Shear, National Cancer Institute to whom the author and his director Dr. Makino express their appreciation.

red granules in their cytoplasm. But a slight agglutination of neutral red granules was observed in the dividing tumor cell after damage. It was easy at this stage to distinguish the undamaged cells from the damaged ones, because the former showed weaker affinity for neutral red than the latter. The frequency in occurrence

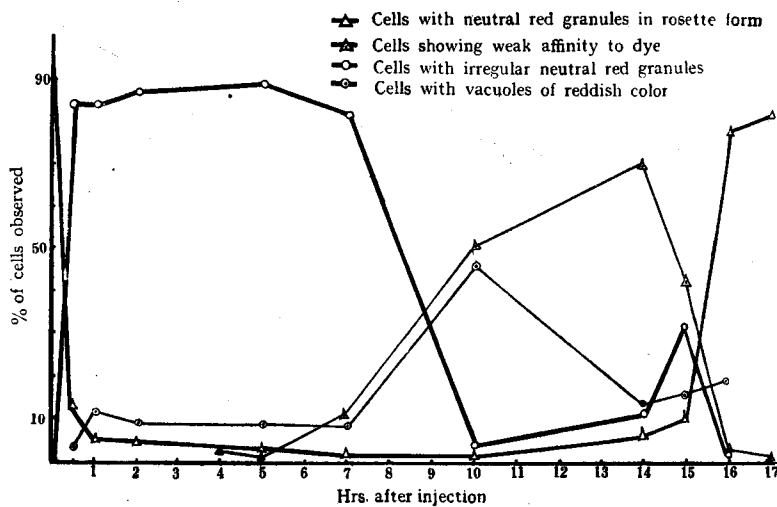


Fig. 11. Graphical representation showing damage of tumor cells induced with α -peltatin treatment in the MTK-sarcoma II (neutral red preparations).

of the R_2 -type cells gradually increased showing the highest frequency about 3 hours after injection. This condition continued for about 8 hours after injection. At about 10 hours there was a remarkable decrease of damaged cells, and a striking appearance of dividing tumor cells which had remained undamaged. Then the cells of the R_1 -type increased in number with time. From 15 to 20 hours after injection, the tumor ascites showed many cells which contained granules arranged in a rosette form in the cytoplasm, together with the cells of the R_2 -type.

Makino (1952), and Makino and Tanaka (1953) suggested that, following exposure to injurious conditions, some of stemline-cells are able to protect themselves by transforming into the resting resistant form, and that possibly, this transformation may be accomplished by the cells becoming impermeable to the noxious substance in the surrounding medium. The weak affinity of undamaged cells for neutral red seems to be concerned with this feature: probably the decrease of staining ability may be explicable as the result of the impermeable nature of the tumor cells. Thus, the cells with weak affinity for neutral red in this experiment are no other than the resistant stemline-cells which have remained

unaffected in the treatment with the drug.

Concluding remarks

From the results obtained in this study, it may be reasonable to suggest that the morphological features of the cytoplasmic granules of the MTK-sarcoma II are different from those of the monocyte, and further that the cytoplasmic granules seem to play an important role in the metabolic activity of the tumor. This was shown, for instance, by the data concerning the frequency distribution of the cells of R_1 - and R_2 -types through a transplant generation (Fig. 9).

Tobioka and Ueoka (1950) studied the correspondence between the behavior of the cytoplasmic granules of the tumor cells and their changes in growth environments with the Yoshida sarcoma by means of supravital double staining with janus green and neutral red. Their results showed that the granules stained with janus green and neutral red appeared most markedly in the tumor cells sampled 2 to 3 days after transplantation, while, nearing the end of life of the tumor-bearing rat, they were replaced by cells not showing these granules. On this basis they suggested the existence of some relationship between cellular function and cytoplasmic granules. The results of this study substantiate the view of Tobioka and Ueoka (1950). Shear and Belkin (1937) attempted a chemical test of living and non-living cells of mouse tumor with neutral red preparations.

Makino and Kanô (1951) have reported in the Yoshida sarcoma that the mitotic rate of the stemline-cells observed with daily material through a transplant generation formed a peak curve through the middle part, decreasing gradually towards the latter part of the life span. The results of the present study on the MTK-sarcoma II revealed that the number of R_1 -type cells showed a gradual increase from the early part continuously towards the middle part of the transplant generation, and then gradually decreased towards the latter part. From a comparison in the daily frequency of the R_1 -type cells with that of the mitotic stemline-cells, it is apparent that the two sets of data run closely parallel, and therefore that the R_1 -type cells are the stemline-cells.

The author's view that the cells of the R_2 -type are those in process of degeneration has been supported by the results of Amano and Hirata (1943), and Amano (1943) from the supravital observations of monocytes. They observed in degenerating monocytes neutral red granules with indefinite and irregular outline which are comparable to those in the R_2 -type cells.

Makino (1952), and Makino and Kanô (1953) have demonstrated that in several rat ascites tumors the stemline-cells are characterized by remarkable ideograms which are specific to each tumor, distinct from the somatic chromosome constitution of rats, and persistent in their individuality through serial transfers. Further, Makino and Tanaka (1953) indicated by treatment with podophyllin that some of the stemline-cells have remained unaffected by the action of podophyllin, and that they constituted the primary source of the subsequent growth of the

tumor. Makino and Tanaka (1953) suggested that the resting stemline-cells are able to resist unfavorable conditions of the surrounding medium by transforming into a small-sized, resistant form, the surface of which may become less permeable to noxious substances. The treatment with α -peltatin in the present study resulted in finding that some of the R_1 -type cells remained undamaged by the drug and showed weak affinity to dye. The weak affinity shown by those undamaged cells may be explained as an expression of the impermeable nature of those cells. It can be said therefore that the tumor cells showing weak affinity to dye are no other than the resistant stemline-cells which have remained alive through the period of the treatment with the chemical.

Summary

The present paper describes the results of investigations of cytoplasmic granules in the tumor cells of the MTK-sarcoma II, which were carried out with supravital staining technique with neutral red.

With regard to the manner in which granules took the stain, the tumor cells were classified into two types as follows :

1) R_1 -type ; the cells of this type contain neutral red granules which are arranged in a rosette form in the cytoplasm. Occasionally, the granules can be seen colored light-green.

2) R_2 -type ; the cells belonging to this type show vacuoles of varying sizes stained with neutral red, together with those of reddish or light-green color, rather indefinite in shape.

Close observations of individual cells in the daily material through a transplant generation of the tumor, and the treatments with a mitotic poison (α -peltatin) revealed that the cells of the R_1 -type are the stemline-cells from which the tumor develops itself, and that the cells of the R_2 -type are the tumor cells which are in process of disintegration.

From the results of this study the suggestion was made that the cytoplasmic granules demonstrated by neutral red seem to play an important rôle in the metabolic activity of tumor cells.

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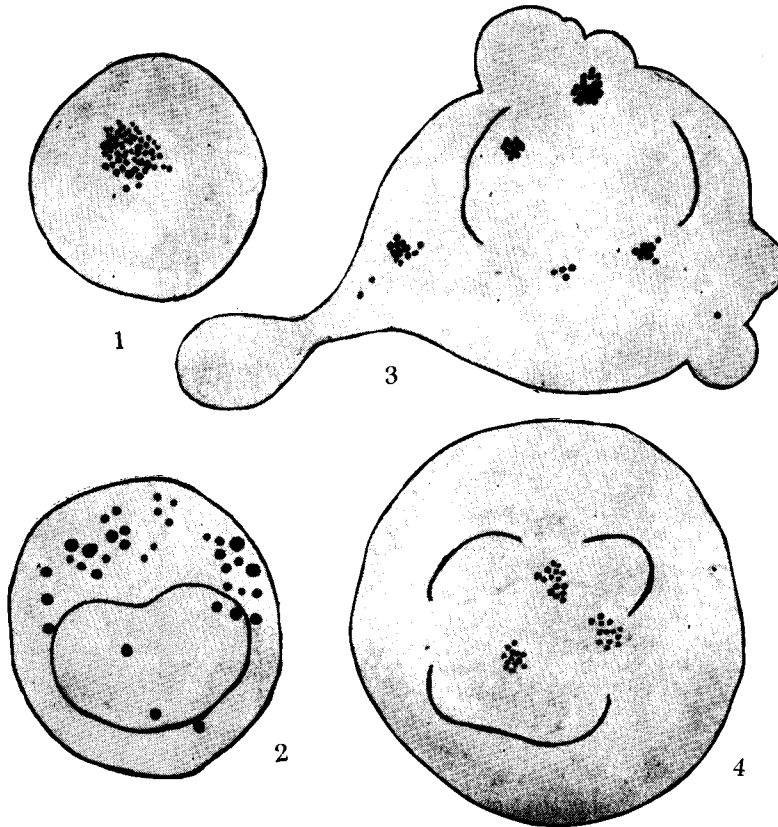
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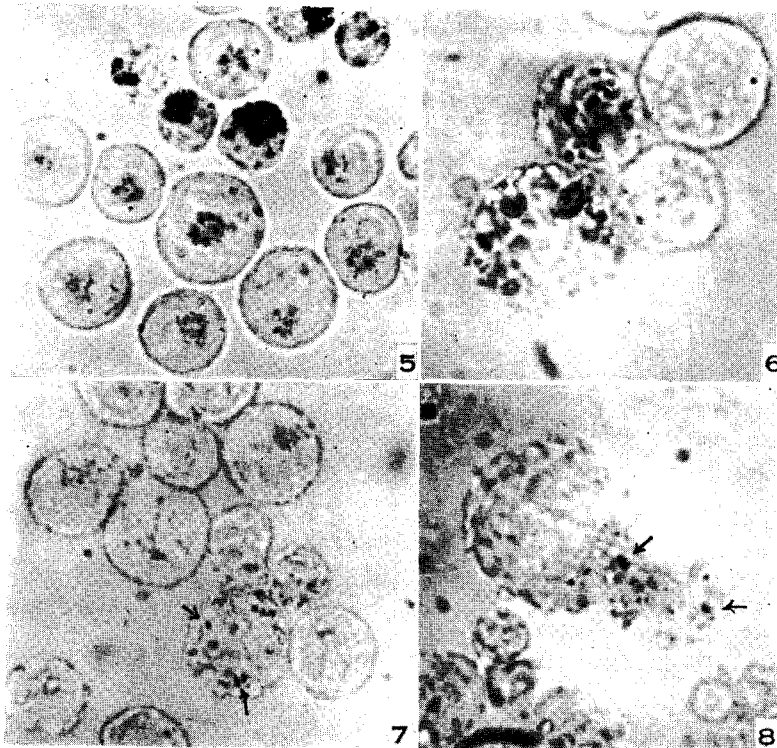


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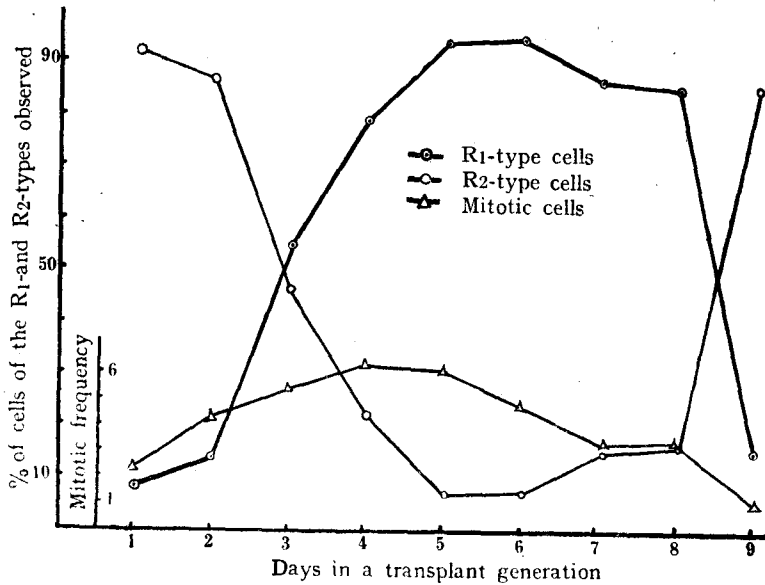


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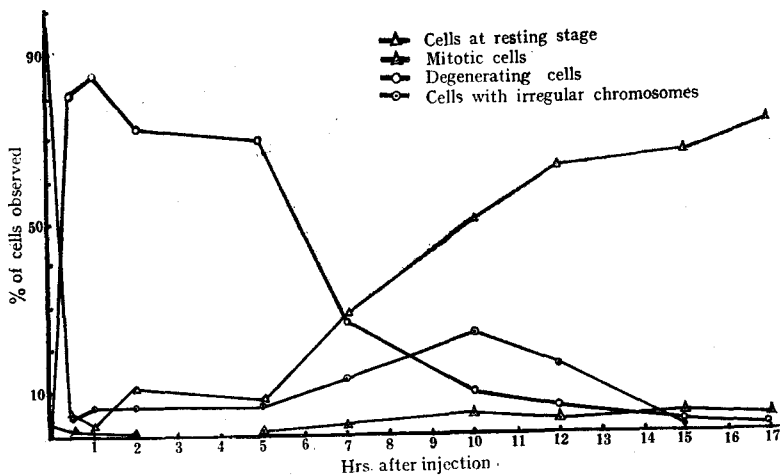


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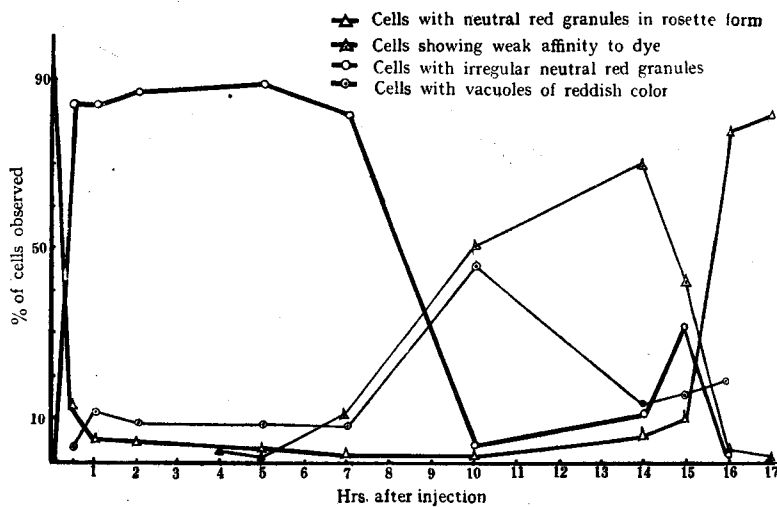


Fig. 11. Graphical representation showing damage of tumor cells induced with α -peltatin treatment in the MTK-sarcoma II (neutral red preparations).

of the R_2 -type cells gradually increased showing the highest frequency about 3 hours after injection. This condition continued for about 8 hours after injection. At about 10 hours there was a remarkable decrease of damaged cells, and a striking appearance of dividing tumor cells which had remained undamaged. Then the cells of the R_1 -type increased in number with time. From 15 to 20 hours after injection, the tumor ascites showed many cells which contained granules arranged in a rosette form in the cytoplasm, together with the cells of the R_2 -type.

Makino (1952), and Makino and Tanaka (1953) suggested that, following exposure to injurious conditions, some of stemline-cells are able to protect themselves by transforming into the resting resistant form, and that possibly, this transformation may be accomplished by the cells becoming impermeable to the noxious substance in the surrounding medium. The weak affinity of undamaged cells for neutral red seems to be concerned with this feature: probably the decrease of staining ability may be explicable as the result of the impermeable nature of the tumor cells. Thus, the cells with weak affinity for neutral red in this experiment are no other than the resistant stemline-cells which have remained

unaffected in the treatment with the drug.

Concluding remarks

From the results obtained in this study, it may be reasonable to suggest that the morphological features of the cytoplasmic granules of the MTK-sarcoma II are different from those of the monocyte, and further that the cytoplasmic granules seem to play an important role in the metabolic activity of the tumor. This was shown, for instance, by the data concerning the frequency distribution of the cells of R_1 - and R_2 -types through a transplant generation (Fig. 9).

Tobioka and Ueoka (1950) studied the correspondence between the behavior of the cytoplasmic granules of the tumor cells and their changes in growth environments with the Yoshida sarcoma by means of supravital double staining with janus green and neutral red. Their results showed that the granules stained with janus green and neutral red appeared most markedly in the tumor cells sampled 2 to 3 days after transplantation, while, nearing the end of life of the tumor-bearing rat, they were replaced by cells not showing these granules. On this basis they suggested the existence of some relationship between cellular function and cytoplasmic granules. The results of this study substantiate the view of Tobioka and Ueoka (1950). Shear and Belkin (1937) attempted a chemical test of living and non-living cells of mouse tumor with neutral red preparations.

Makino and Kanô (1951) have reported in the Yoshida sarcoma that the mitotic rate of the stemline-cells observed with daily material through a transplant generation formed a peak curve through the middle part, decreasing gradually towards the latter part of the life span. The results of the present study on the MTK-sarcoma II revealed that the number of R_1 -type cells showed a gradual increase from the early part continuously towards the middle part of the transplant generation, and then gradually decreased towards the latter part. From a comparison in the daily frequency of the R_1 -type cells with that of the mitotic stemline-cells, it is apparent that the two sets of data run closely parallel, and therefore that the R_1 -type cells are the stemline-cells.

The author's view that the cells of the R_2 -type are those in process of degeneration has been supported by the results of Amano and Hirata (1943), and Amano (1943) from the supravital observations of monocytes. They observed in degenerating monocytes neutral red granules with indefinite and irregular outline which are comparable to those in the R_2 -type cells.

Makino (1952), and Makino and Kanô (1953) have demonstrated that in several rat ascites tumors the stemline-cells are characterized by remarkable ideograms which are specific to each tumor, distinct from the somatic chromosome constitution of rats, and persistent in their individuality through serial transfers. Further, Makino and Tanaka (1953) indicated by treatment with podophyllin that some of the stemline-cells have remained unaffected by the action of podophyllin, and that they constituted the primary source of the subsequent growth of the

tumor. Makino and Tanaka (1953) suggested that the resting stemline-cells are able to resist unfavorable conditions of the surrounding medium by transforming into a small-sized, resistant form, the surface of which may become less permeable to noxious substances. The treatment with α -peltatin in the present study resulted in finding that some of the R_1 -type cells remained undamaged by the drug and showed weak affinity to dye. The weak affinity shown by those undamaged cells may be explained as an expression of the impermeable nature of those cells. It can be said therefore that the tumor cells showing weak affinity to dye are no other than the resistant stemline-cells which have remained alive through the period of the treatment with the chemical.

Summary

The present paper describes the results of investigations of cytoplasmic granules in the tumor cells of the MTK-sarcoma II, which were carried out with supravital staining technique with neutral red.

With regard to the manner in which granules took the stain, the tumor cells were classified into two types as follows :

1) R_1 -type ; the cells of this type contain neutral red granules which are arranged in a rosette form in the cytoplasm. Occasionally, the granules can be seen colored light-green.

2) R_2 -type ; the cells belonging to this type show vacuoles of varying sizes stained with neutral red, together with those of reddish or light-green color, rather indefinite in shape.

Close observations of individual cells in the daily material through a transplant generation of the tumor, and the treatments with a mitotic poison (α -peltatin) revealed that the cells of the R_1 -type are the stemline-cells from which the tumor develops itself, and that the cells of the R_2 -type are the tumor cells which are in process of disintegration.

From the results of this study the suggestion was made that the cytoplasmic granules demonstrated by neutral red seem to play an important rôle in the metabolic activity of tumor cells.

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