Cytological Effects of Chemicals on Tumors, XXVIII Notes on the Effect of Extract from *Paederus fuscipes* on a Transplantable Rat Ascites Tumor[1),2),3)]

By

Yohko Hisada and Makito Emura

Zoological Institute, Hokkaido University

(With 21 Text-figures and 1 Table)

The existence of a toxic substance in various species of insects belonging to the genus *Paederus* (Staphylinidae, Coleoptera) has been described by many investigators (Netolitzky, 1919; Gordon, 1925; Pavan and Bo, 1953; Matsumoto, 1964; and so on), with particular concern to physiological and chemical properties. Pavan (1963) seems to be the first who dealt with its biological activity, by working on possible influence of the toxic substance (pederin) derived from *Paederus fuscipes* on tumor fragments (sarcoma 180): he reported that 'pederin' reduced or completely prevented tumor growth and that it acted as an antimitotic, provoking chromosomal alterations and blocking the formation of the spindle at metaphase. He and his co-workers noted a possible biological effect of pederin on animal tissues as necrotizing and stimulating agent. The present paper deals with a cytological study on the antitumor action of pederin, use being made of crude methanol extract from *Paederus fuscipes*.

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Materials and methods: Methanol extract from *Paederus fuscipes* containing about 50 per cent of pederin was provided by Dr. Matsumoto of Chemistry Department. Tumor

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cells used for the present study were exclusively MTK-sarcoma III, a transplantable rat ascites tumor. Rats of Wistar strain weighing 80 to 180 grams were used as the hosts for tumor transfers. Stock extract was dissolved in 50 per cent ethanol at various concentrations, and the solution was diluted with sterilized distilled water to give final concentrations of 0.125, 0.25, 0.5 and 1.0 mg in one ml of 2 per cent ethanol solution. The treatment started on the third day of tumor transfer. Samplings were made at 4-hour intervals in the first 28 hours after treatment, and from then on at 12-hour intervals, so long as the animals were alive.

Samplings were also made at shorter intervals in particular cases in the course of study. For the preparation of slides the following methods were adopted: methyl violet (MV) squash method for general cytological observations, May-Grünwald-Giemsa (MGG) staining method (Jacobson and Webb 1952) for particular structural changes, phase microscopy for living material, and Gomori's acid phosphatase staining method (Gomori 1952) for lysosomes. At the same time autoradiography with H^3-thymidine was applied in order to examine possible effects of the extract on DNA synthesis of tumor cells.

Results

To obtain an adequate dosage less toxic to hosts but more effective on the tumor cells, suspensions at several concentrations as above noted were tested by administering them to the tumor-bearing animals at a dose of 1 ml per 100g body weight (Table 1). As a result, 0.125 mg/100g body weight was chosen for an adequate dosage for carrying out the present cytological examinations. Within 1 hour after application pycnosis of nuclei occurred in the affected leucocytes as the first remarkable effect. From then on, tumor cell damages became progressively evident (Figs. 2–5). The damages were represented by chromosomal alterations in mitotic cells, as well as by the nuclear and cytoplasmic disintegration in interphase cells. Details are presented in the following.

Table 1. Life span of tumor-bearing rats (MTK-sarcoma) after treatment with extract from *Paederus fuscipes*

Dose (mg/100g body weight)	Body weight (g)	No of animals	Host survival time after treatment (hour)	Life span (day)
1	80-180	5	5.4 (4- 6.5)	3
0.5	90-160	5	6.1 (5- 10.5)	3
0.25	110-140	5	19.2 (10- 30)	3
0.125	80-180	20	83.1 (30-185)	6
Control	80-120	10	—	8

1) *Mitotic cells:* As shown in Figure 1a, a marked effect was found after exposure for 4 hours. Within 2 to 8 hours of treatment, it was observed that,

though some of dividing cells completed the division, many cells were arrested at metaphase in abnormal condition. Some abnormal cells showed contracted chromosomes scattering in the cytoplasm (Fig. 6). With the passage of time, metaphase-arrested cells increased in number and underwent disintegration. In 12-hour-samples no anaphase and telophase figures occurred, and 24-hour-samples showed no prophase cells. Phase microscopy study revealed that, in some cases, metaphasic cells formed the cleavage furrow, but cell division was not completed. Similar feature was presented by Makino and Nakanishi (1955) in the experiment with podophyllin. 28-hour-specimens showed mitotic cells with dense masses of chromosomal materials (Figs. 7 and 9). Figures 7 and 8 indicate clearly the contraction of metaphase chromosomes together with some pycnotic ones. The mitotic index gradually decreased and the mitotic cells completely disappeared during a period from 36 to 78 hours after application of the agent.

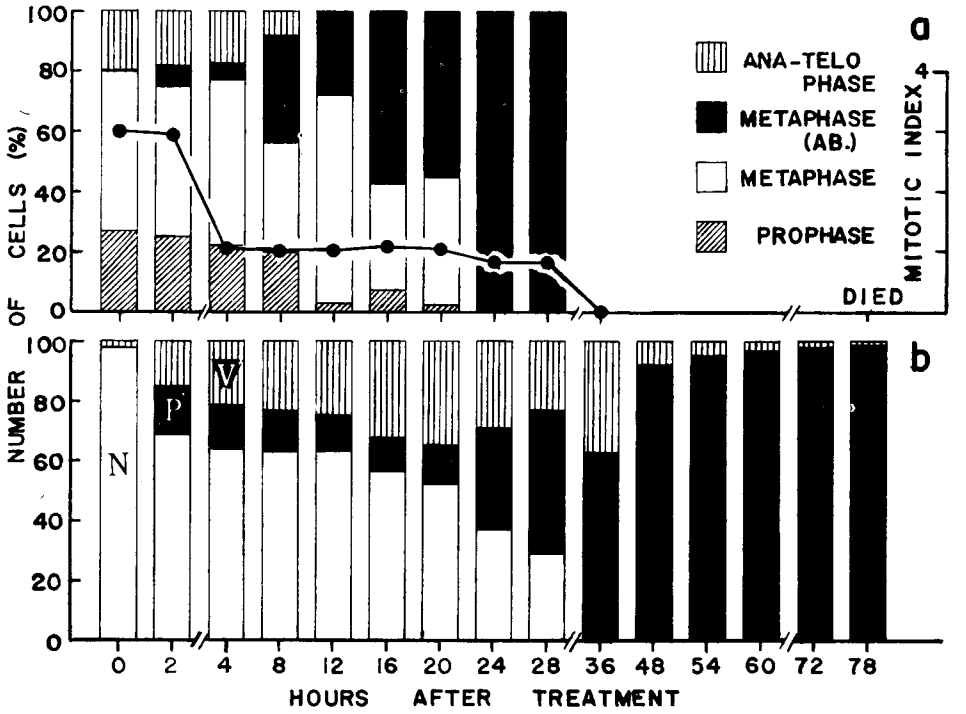
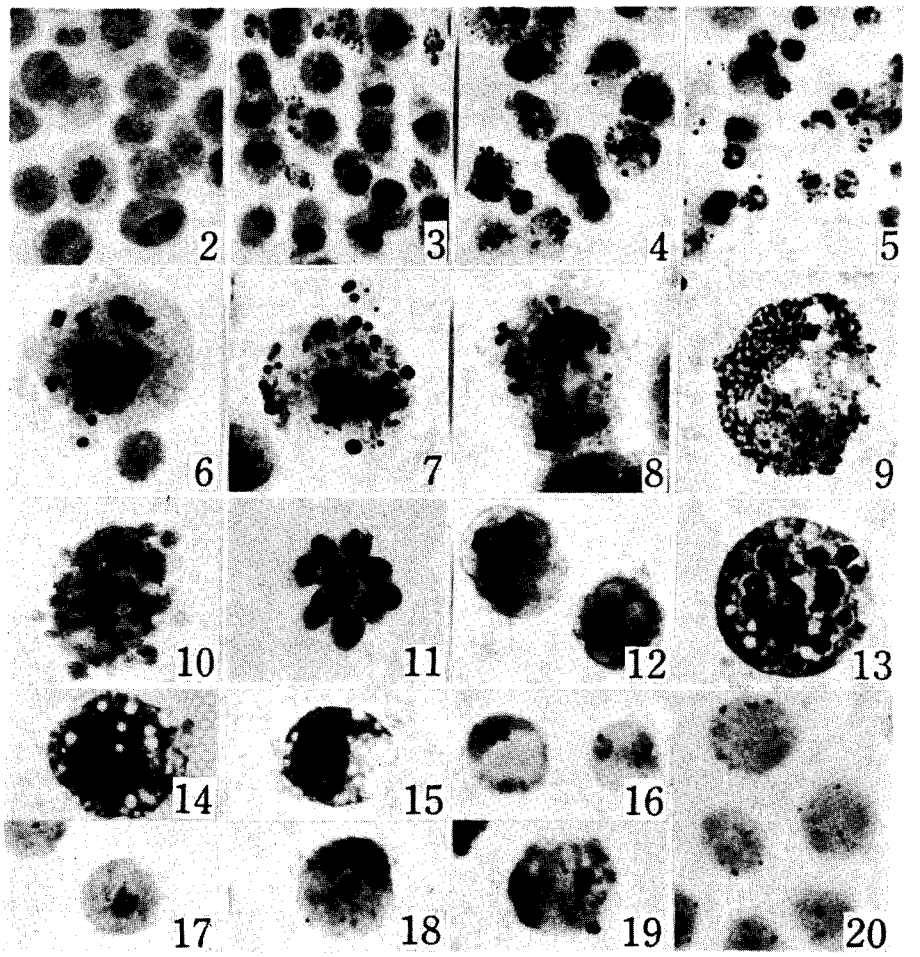


Fig. 1. Graph showing effect of extract. a, Curve showing mitotic index of tumor cells. b, Histograms showing relative frequencies of unaffected and affected cells. N: Normal cell, P: Pycnotic cell, V: Vacuolized cell.

2) *Interphase cells:* The effect of this extract appeared in interphase cells within 2 hours of treatment. As shown in Figure 1b, about 30 per cent of the



Figs. 2–20. Degenerative features of MTK-sarcoma III cells following the extract treatment. 2, Untreated control. 3–5, Advanced condition of cell degeneration (MV). 3, 2 hrs. 4, 28 hrs. 5, 72 hrs. 6, Contraction of chromosomes at metaphase cell (MV), 19 hrs. 7–8, Metaphase cells showing contracted chromosomes and pycnotic chromosomal masses (MV), 14 hrs. 9, Pycnotic substances dispersed and vacuolizations in cytoplasm (MGG), 56 hrs. 10, Blebbing of cytoplasm (MV), 4 hrs. 11, Blebbing of cytoplasm and dense chromatin substances (MV), 48 hrs. 12, Swelling of nucleoli and nuclei (MV), 8 hrs. 13, Pycnotic cell (MGG), 30 hrs. 14, Vacuolization of nuclei and cytoplasm (MGG), 30 hrs. 15, Residual cell (MGG), 72 hrs. 16–20. Showing localizations of lysosomes in untreated and treated cells (after Gomori's acid phosphatase method). 16, Polarized lysosomes on both sides of the nucleus. 17, Lysosomes occupying the central part of the cell. 18, Lysosomes distributed over the whole cell. 19, A cytolysome in the cell, 32 hrs. 20, "Circumference" disposition of lysosomes in cells, 14 hrs.

interphase cells showed disorganization, represented by aggregation of the chromatin substance and vacuolization of the cytoplasm (Figs. 13 and 14). The swelling and vacuolization of nuclei and blebbing of the cytoplasm were remarkable as morphological changes (Figs. 10–12). By 36 hours, the vast majority of tumor cells were affected and consequently pycnotic nuclei were dominant, being approximately 99 per cent in occurrence. The remaining cells showed also degenerative configurations with vacuoles in the cytoplasm, being well-demonstrated in MGG preparations (Fig. 15). In samples exposed for 88 and 96 hours, a great number of cells were in process of disintegration. At that time the cells were transferred into the peritoneal cavity of new hosts. Tumor recovery occurred in them during 8 to 10 days in 5 out of 15 cases.

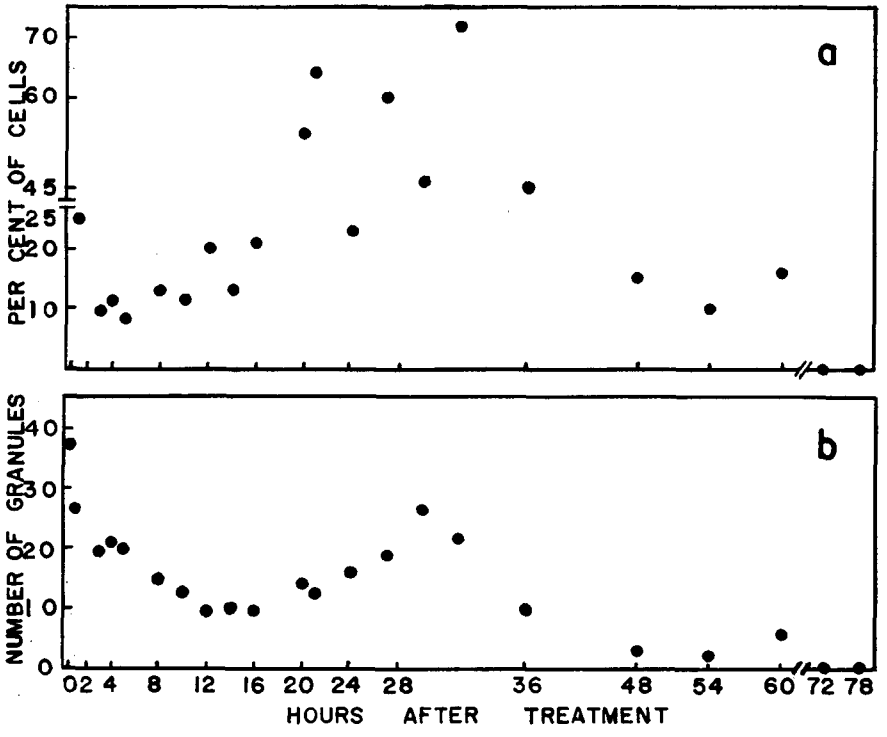


Fig. 21. Effect of the extract from *Paederus fuscipes* on lysosomes in the MTK-sarcoma III cells, expressed as a function of time after treatment. a. Frequencies of tumor cells carrying cytolysomes. The ordinate is defined as the percentage of the tumor cells with at least one cytolysome. b. Changes in number of lysosomal granules. The ordinate represents the average per cell number of granules.

3) *Lysosomes:* a) Based on the localization of the lysosomes, untreated tumor cells were classified into three types as follows: cells of the first type contain the granules polarizing juxtanuclearly on both sides of nuclei (Fig. 16); in the second-type cells, the granules assemble in the central part of the cytoplasm (Fig. 17), and the third type cells show the granules distributing evenly over the whole cytoplasm (Fig. 18). It was observed that approximately 50 per cent of the untreated cells were of the second type, whereas 30 per cent and 20 per cent of the untreated cells were the third and the first types, respectively. The cells of three types decreased in number with time, and they disappeared by 8 to 10 hours after treatment, when another type of cells made their appearance (Fig. 20). Localization pattern of the lysosomes in those cells was similar to that of the metaphasic or otherwise colchicine treated interphasic cells of HeLa strain, the lysosomes of which were circumferentially disposed (Robbins and Gonatas, 1964).

b) Number and shape of the granules stained for acid phosphatase: the decrease in number of granules per cell persisted for about 16 hours after treatment. Then, the number increased and reached a maximum by 30 hours after injection. From then on, the number of granules decreased rapidly without restoration (Fig. 21b). Occurrence of the "cytolysomes" (Fig. 19; cf. Novikoff, 1960) was noted throughout the course of treatment (Fig. 21a). Cells with cytolysomes reached a maximum in number at about 30 hours or more after treatment, then decreased quickly. In the cells both untreated (Figs. 16–18) and treated (Fig. 20) for 1 to 16 hours, smaller granules of rather uniform shape were commonly observed.

4) *Autoradiography:* After 27 hours of treatment when most of the cells seemed to have been affected severely by the extract, H^3-thymidine (10μc per 0.3 ml) was injected intraperitoneally into the hosts. Two to four hours after the H^3-thymidine treatment, samples were removed from the hosts. The cells of each sample were immediately treated from the hosts. The cells of each sample were immediately treated with hypotonic solution, and fixed in acetic alcohol (1 : 3). Slides were prepared according to air-dry technique (Moorhead *et al.*, 1960), with carbol fuchsin staining (Carr and Walker, 1961). Then the slides were dipped in Sakura liquid emulsion and exposed for a week at 4°C. The samples examined so far showed no interphase nuclei labeled with the precursor during the whole period of the exposure to H^3-thymidine, while untreated interphase nuclei of MTK-sarcoma III cells were found to be labeled within 1 hour.

Discussion

Pavan (1963) reported that pederin had inhibitory and stimulatory properties for cell growth. A stimulatory property of this agent was shown by Deotto (cited from Pavan, 1963) in the development of a hepatectomized rat liver. Pavan and his co-workers, based on cytological study with the meristem of *Allium cepa*, suggested an antimitotic nature of pederin. The present study indicated that metaphase cells were constant in frequency, while prophase cells showed a remarkable

decrease, and that the decline of the mitotic index was striking. In virtue of the above facts, it may be possible to assume that this extract damages directly the interphase cells, or prevent them from entering mitosis. Further, the process from metaphase to anaphase seems also to be inhibited, and prophase cells remain unaffected. The above assumption may be supported by the constant frequency value of normal metaphase cells along with a rapid decrease of ana-telophase cells. A rapid increase in frequency of total metaphase cells including abnormal metaphase cells may also be an indication that the metaphase-anaphase process is inhibited. Phase microscopy study revealed that cell division was not completed, while the formation of a cleavage furrow occurred. Similar features were provided by several authors with the application of various mitotic poisons (Makino and Cornman, 1953; Makino and Nakanishi, 1955; George *et al.*, 1965). It seems very probable that this extract acts to some extent as a mitotic poison, capable of suppressing mitotic activity of interphase as well as metaphase cells. It has been reported that cytolysomes are formed in a variety of cells undergoing lysis (Novikoff, 1959; 1960; Barron, 1961; Barron and Sklar, 1961; Becker and Barron, 1961; Novikoff and Essner, 1962), and that lysosomes become circumferentially disposed with the application of spinlde inhibitors (Robbins and Gonatas, 1964). The intense activity of acid phosphatase indicated by the cytolysomes and the maximum number of lysosomes occurring in 28- to 36-hour samples (Figs. 21a and b) may possibly indicate that a large number of tumor cells are undergoing disintegration. Further, it is possible that the circumferential lysosomal disposition observed in almost all the interphase cells treated with the extract is an effect secondary to an inhibition of the synthesis of microtubular constitutents and/or a destruction of microtubules themselves, as suggested by Robbins and Gonatas (1964). Since it is now known that microtubule structures are present in most, if not all, interphase cells of varying types (Slautterback, 1963; Robbins and Gonatas, 1964), and it has been suggested that they partake in the direction of protoplasmic streaming (Ledbetter and Porter, 1963), it is highly probable that the disintegration of microtubular arrays in interphase would prevent the protoplasmic streaming, and that the structural depolarization of the lysosomes would result from simple ever-present Brownian movement.

Almost complete regression of ascites tumors occurred after a long period of treatment with this extract. No incorporation of H^3-thymidine prevailed in nuclei of morphologically normal interphase cells that had survived long treatment with the extract, during which mitosis has been completely suppressed. On the other hand, untreated interphase tumor cells incorporate a significant amount of the precursor within 2 hours (Kurita *et al.*, 1964; Hisada unpublished). It is thus apparent that the extract causes an inhibition of thymidine incorporation. Following the treatment, some interphase cells, when transferred into a new host, showed vigorous proliferation. This indicates that in some interphase cells, the DNA synthesizing system has been simply repressed but not damaged. In the light of the

above findings, a conclusion may be possible that pederin prevents in cells the process from metaphase to anaphase, together with a variety of destruction such as vacuolization, and blebbing in both cytoplasm and nuclei.

In this report a facet of action of pederin as an inhibitory agent for cell multiplication was shown. It will be an interesting problem to answer the question as to why pederin can exerts such an outwardly incompatible effects, an inhibitory and a stimulatory one, for tissue growth as suggested by Pavan.

Summary

Damaging effects of crude methanol extract from *Paederus fuscipes* on an ascites sarcoma of rats were cytologically examined. It exerted a considerable influence upon both interphase and mitotic cells, inhibiting mitosis. The influence induced a variety of morphological transformations. They are represented by contraction, pycnosis and disorientation of the chromosomes, karyorrhexis, karyopycnosis, vacuolization, blebbing of cellular elements, and several lysosomal alterations. The application of autoradiography revealed that cells showed no incorporation of H^3-thymidine at all after 27 hours of the extract treatment. But the inoculation of those cells in new hosts resulted in tumor growth in a few cases.

References

Barron, K.D. 1961. Histochemical study of neuronal necrosis produced by intracerebral injection of diphtheria toxin. Neurology **11**: 714–723.

————, and S. Sklar 1961. Response of lysosomes of bulbospinal motoneurons to axon section. Neurology **11**: 866–875.

Carr, D.H., and J.E. Walker 1961. Carbol fuchsin as a stain for human chromosome. Stain Technol. **36**: 233–236.

George, P., L.J. Journey, and M.N. Goldstein 1965. Effect of Vincristine on the fine structure of HeLa cells during mitosis. J. Nat. Cancer Inst. **35**: 355–375.

Gomori, G. 1952. "Microscopic Histochemistry: Principles and Practice". Univ. Chicago Press, Chicago, Illinois.

Gordon, M.R. 1925. A note on two vesicant beetles belonging to the family Staphylinidae. Ann. Trop. Med. Parasit. **19**: 47–52.

Jacobson, W., and M. Webb 1952. The two types of nucleoproteins during mitosis. Exp. Cell Res. **13**: 163–183.

Kurita, Y., K. Moriwaki, and T.H. Yosida 1964. Autoradiographic analysis of the mitotic cycle in Yoshida sarcoma cells. Gann **55**: 397–402.

Ledbetter, M.C., and K.R. Porter 1963. A "microtubule" in plant cell fine structure. J. Cell Biol. **19**: 239–250.

Matsumoto, T., S. Tsutsui, M. Yanagiya, S. Yasuda, S. Maeno, J. Kawashima, A. Ueta, and M. Murakami 1964. The partial structure of pederin. Bull. Chem. Soc. Japan **37**: 1892–1893.

Moorhead, P.S., P.C. Nowell, W.J. Mellman, D.M. Battips, and D.A. Hungerford 1960. Chromosome preparations of leukocytes cultured from human peripheral blood. Exp. Cell Res. **20**: 613–616.

Makino, S., and I. Cornman 1953. Different cytological responses of malignant cells to

podophyllotoxin *in vitro*. Cytologia **18**: 277–282.

Makino, S., and Y.H. Nakanishi 1955. The cytological effect of chemicals on ascites sarcoma, IV. A phase microscopy study of the tumor cells by podophyllin. Cytologia **20**: 89–95.

Netolitzky, F. 1919. Fine neue Gruppe blasenziehender Käfer aus Mitteleuropa (*Paederus*, Staphylinidae). Zeitschr. f. Ang. Entom **5**: 252–257.

Novikoff, A.B. 1959. Biochemical and staining reactions of cytoplasmic constituents. in "Developing Cell Systems and Their Control" Ronald Press, New York.

————, and E. Essner 1962. Cytolysomes and mitochondrial degeneration. J. Cell Biol. **15**: 140.

Pavan, M. 1963. Ricerche Biologiche e Mediche su Pederina e su Estratti Purificati di *Paederus fuscipes* Curt. (Coleoptera Staphylinidae). Pavia.

————, and G. Bo 1953. Pederin, toxic principle obtained in the crystalline state from the beetle *Paederus fuscipes* Curt. Phys. Comp. et Oecol. **3**: 307–312.

Robbins, E., and N.K. Gonatas 1964. Histochemical and ultrastructural studies on HeLa cell cultures exposed to spindle inhibitors with special reference to the interphase cell. J. Histochem. Cytochem. **12**: 704–711.

Slautterback, D.B. 1963. Cytoplasmic microtubules I. Hydra. J. Cell Biol. **18**: 367–388.