Cytological Studies on Cancer, IV. General Characters of the MTK-Sarcomas, New Ascites Tumors of Rats produced by the Administration of Azo Dye[1)]

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(With 8 Text-figures and 1 Plate)

Recent interest in the field of cancer pathology has arisen around the fluid tumor. Several attempts have been made to transform a malignant tumor into a fluid form by certain investigators, such as Hesse (1927), Loewenthal (1932), Takizawa (1948), and Goldie and Felix (1950). The so-called ascites tumor was produced by these authors by injecting the tumor emulsion into the abdominal cavities of rats or mice.

Recently, Yoshida, with the co-operation of his followers (1944, 1949a, b), succeeded in establishing a real fluid tumor generally called the Yoshida sarcoma. This is an ascites tumor which grows malignantly in the peritoneal cavity of white rats (*Rattus norvegicus*) in the form of fluid tumor ; it is capable of successive transplantations. According to Yoshida (1949), the Yoshida sarcoma originally developed in a white rat which had been fed with o-Aminoazotoluene for 3 months and then applied cutaneously with potassium arsenite solution for about 4 months. Such a peculiar tumor as the above never been reported by any author. Among the investigators there has been a question whether the Yoshida sarcoma had been produced spontaneously or induced by o-Aminoazotoluene. Yoshida and his followers have made repeated attempts to produce another ascites sarcoma but in vain. Thus, the origin of the Yoshida sarcoma has been left in question.

Tanaka, one of the present authors, has been engaged in the development of the hepatoma by the administration of azo dye for the purpose of the cytological research under the guidance of Professor Makino. In the course of this experiment,

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This article constitutes one of the serial studies having been done by S. Makino and his followers. (S.M.).

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the authors found in two rats the development of an ascites tumor similar in nature to the Yoshida sarcoma. The essential points in this case were reported to the 10th Annual Meeting of the Japanese Cancer Association, April 3, 1951, Tokyo (Makino, Tanaka and Kanô 1951). The present paper describes in detail the procedure of the experiment, with a report on some observations of the cytological features of these ascites tumors in comparison with the Yoshida sarcoma.

The albino rats (*Rattus norvegicus*) used as material for the experiment are derivatives of the Wistar strain which have been bred pure in our laboratory. Full-grown rats, 100-120 gr in body weight, were employed. Eighty rats were administrated o-Aminoazotoluene (O. A. T.) for 300 days accroding to the method of hepatoma-producing-experiment by Sasaki and Yoshida (1935). After this treatment, some of these rats have been fed on p-Dimethylaminoazobenzene (D.A.B.) according to the method of Harada et al (1937). The total mass of the dye administrated were calculated from the weight of the remainder of the diet. Microscopical observations have been carried out with the smear preparations stained with Giemsa's stain, acetic gentianviolet and acetocarmine.

The authors wish to express their sincere gratitude to Professor Sajiro Makino under whose guidance the work has been carried out, for his valuable suggestions given at every time and kindness in revising the manuscript for publication. Further, thanks are extended to Professor Tomizo Yoshida for his valuable criticisms and kind help by supplying carcinogenic agents. To Dr. Haruo Sato, Dr. Hiroshi Sato, and Dr. Tosihide H. Yosida the authors are also greatly indebted for their kind aid in various ways.

I. Procedure of experiments and the development of new ascites tumors

Eighty white rats have been administrated with O.A.T. Since the main subject of this study was to investigate the cytological phenomena during carcinogenesis in livers of rats, the animals were sacrificed for autopsy at necessary intervals. However, fifteen individuals were left alive to observe the condition of development of hepatoma. In about 300 days after treamtent, the remarkable development of malignant hepatoma had occurred in the livers of six of those fifteen animals, while the remaining nine animals showed no evidence for the malignant gorwth of liver. Then, one of them was killed for autopsy. The liver of this animal showed the so-called atrophic lesion giving dark brown colouration. But, no histological study of this tissue and microscopical investigation of the ascites was made.

Referring to the paper of Harada et al (1937), Tanaka, one of the authors, gained the impression that the application of p-Dimethylaminozaobenzene (D.A.B.) would bring about some additional effects to induce the tumor, due to its striking carcinogenic action. Therefore, the administration of O.A.T. was stopped, and the application of D.A.B. was continued according to the method of Harada et

al (1937). Meanwhile, in one of eight animals thus treated, an unexpected neoplastic growth was detected in the left abdominal cavity after 30 days of the D.A.B. application. This feature of left abdomen simulated that of the early hepatoma stage by showing an expanded appearance commonly found in a tumor infiltrating into surroundings.

Fig. 1. Topographical view of the original tumor animal of the MTK-sarcoma I. Fig. 2. Topographical view of the original tumor animal of the MTK-sarcoma II. *l*; liver *t*; tumor. *s*; spleen.

After 40 days' treatment, the neoplastic growth became increasingly larger in size. The animal showed a diseased condition, so that it was sacrificed for autopsy. In the peritoneal cavity, there was found a mikly ascites of about 20 cc, with some evidence of haemorrhage. The topographical view of this animal is shown in Figure 1. The microscopical observations of the ascites indicated that the milky ascites was a suspension of tumor cells. The tumor cells were distinguishable from monocytes by means of the morphological observations; the former are characterized by the cytoplasm of highly basophilic nature, vague at circumference (Figs. 9-11), with their kidney-shaped nuclei located in one side of the cell, whereas the latter cells are remarkable for their content of lightly stained cytoplasm, sharp at circumference. Moreover, strikingly distinct azurophilic granules arranged in a rosette form occupy a wide area in the cytoplasm. Then the milky fluid, an

emulsion of tumor cells, was transplanted into the peritoneal cavity of eight animals according to the method advised by Sato (cf. Yoshida 1949a, b). The animals thus treated underwent a striking change in the ascites after five days. Namely, the ascites became strikingly increased in the peritoneal cavity, just as in the Yoshida sarcoma, and the abdomen showed a remarkable expansion.

Microscopical observations were carried out with the tumor ascites thus developed. They led to the conclusion that in general features the ascites essentially similar to that of the Yoshida sarcoma, not only in the proliferation of tumor cells contained but also in the morphological characteristics of tumor cells (Figs. 12-14). Up to the present time, succeeding transplantations of the ascites tumor have been continued over 71 generations. Some pieces of tumor tissue were grafted subcutaneously in some rats. They developed into a large massive tumor as in the ordinary sarcoma. The scheme of this experiment is given in Table 1. The strain of the present new tumor will be called "*MTK-sarcoma I*" in the following.

Table 1. Schedule of experiment

MTK sarcoma	Duration of administration of O.A.T.	Total mass of O.A.T. (mg)	Duration of administration of D.A.B.	Total mass of D.A.B. (mg)	Body weight (gr)	Sex	Hepatic exponent*
I	312	2096	41	227	125	♂	—
II	312	1872	103	792	175	♀	14.10

* Hepatic exponent=liver weight/body weight ×100.

Another case of the new ascites sarcoma was also developed in a white rat which was administrated with D.A.B. for 103 days after the O.A.T. treatment. The scheme of the experiment for the establishment of this sarcoma is shown in Table 1. The first detection of abdominal diagnosis was observed on the 51st day of treatment with D.A.B. At that time, there was found in the peritoneal cavity of this animal, a considerable amount of ascites, but it contained no tumor cells or like ones. This condition has continued for about 10 days. The hypertrophy of liver was produced with time. On the 90th day of treatment with D.A.B., a remarkable change occurred in the peritoneal fluid. It was a cange of monocytes; they increased in number due to division on the one hand, and on the other hand showed atrophy, with morphological variations of the nucleus. In response to the numerical increase, the monocytes assumed the appearance of macrophagocytes. Mingled with these monocytes there occurred a few tumor cells containing basophilic cytoplasm. The tumor cells are very prominent in respect to being larger in size than any other cells observable in the peritoneal cavity.

On the 103rd day of treatment, the animal was killed for autopsy, since it was in a diseased condition. Externally this animal showed a neoplastic growth

as shown in Fig. 2. In the peritoneal cavity there was found a cnsiderable amount of ascites, about 30 cc in volume. The microscopical observations disclosed that the ascites was a suspension of tumor cells (Fig. 15). Successive transplantations of those tumor cells have been continued up to the present time from rat to rat for 59 generations. We propose to call this strain of the ascites tumor "*MTK-sarcoma II*" in the following.

II. General characteristics of the MTK-sarcomas as compared with those of the Yoshida sarcoma

The general characteristics of the MTK-sarcomas here concerned were found to be essentially similar to those of the Yoshida sarcoma in every respect, as detalied in the following descriptions.

1) *Tumor cells*: The tumor cells are very prominent for their large size as compared with any other cells observable in the peritoneal cavity. The nuclei are of oval or kidney, or sometimes biloped shape, and are situated on one side of the cell. Conspicuous nucleoli, generally two or more in number, are visible within the nuclear body. In the wide space of the cell, distinct azurophilic granules appear in the cytoplasm arranged in a remarkably rosette form (Figs. 12-14 and 16-17).

2) *Rate of successful transplantation*: For successive transplantations of tumor cells, albino rats of 60-80 gr body weight were employed as the host animals. There was no difference in susceptibility as regards either age or sex.

Table 2. Rate of transplantation nuder comparison in three strains of the ascites tumor

Strains of ascites tumor	Transplant generation	Total number of animals transplanted	Number of tumor animals died	%
Yoshida sarcoma	67	150	132	88.0
MTK-sarcoma I	35	69	59	85.5
MTK-sarcoma II	27	50	44	88.0

In our laboratory the rate of the successful transplantation of the Yoshida sarcoma was 88.0 percent on an average. The rate of transplantation of the MTK-sarcoma I was 85.5 percent (Table 2). At present, the MTK-sarcoma I has attained 71 generations, while the MTK-sarcoma II 59 generations in successive transplantations.

3) *Life span of tumor-bearing animals*: In both MTK-sarcoma I and II, the whole life span extending from the first day of transplantation of tumor ascites to the death of the host animal is rather short, just as in the Yoshida sarcoma. Most of the tumor rats died at about 10 days after transplantation. The average

duration was a little more than 12 days in the MTK-sarcoma I, while it was ten days or more in the MTK-sarcoma II, the extreme case being 20 days. In our laboratory the Yoshida sarcoma rats died at 13 days on the average (Table 3).

Table 3. Life span of the tumor-bearing animals under comparison in three strains of the ascites tumor

Strains of ascites tumor	Total number of tumor animals	Average duration of life span (days)	Maximium duration (days)	Minimum duration (days)
Yoshida sarcoma	100	13.1	27	5
MTK-sarcoma I	53	12.6	28	8
MTK-sarcoma II	36	10.6	21	7

4) *Rate of mitosis in a transplant generation*: Daily observations on the mitotic rate in the Yoshida sarcoma cells were carried out through the whole life span of certain tumor rats (Kanô 1951, Makino and Kanô 1951). The mitotic rate was also observed in the MTK-sarcoma I in the same way as in the Yoshida sarcoma: namely two thousand tumor cells were observed every day through a transplant generation for 12 days, and daily frequencies of all dividing cells being at late prophase, metaphase, anaphase and telophase were calculated in percentage. The results are given in Table 4.

Table 4. Daily frequency of mitotic cells in a tumor rat (MTK-sarcoma I). The percentage of dividing cells was calculated on the basis of 2000 cells per day in the observation through a transplant generation

Days after transplantation	1	2	3	4	5	6	7	8	9	10	11	12
% of dividing cells	1.6	2.7	3.3	3.0	4.0	3.7	3.2	2.3	2.5	1.7	0.5	1.2

In comparison of the data between the MTK-sarcoma I and the Yoshida sarcoma (Chart 1), it is evident that the latter seems to show a frequency higher than the MTK-sarcoma I in the mitotic rate. Generally speaking, the number of dividing cells strikingly increases during the early part of a transplant generation, while the mitotic rate shows the highest frequency during the middle part, that is 5 or 6 days after transplantation. Then, it decreases gradually towards the latter part of the life span.

5) *Abnormal mitosis in tumor cells*: Various mitotic abnormalities were reported in the Yoshida sarcoma by Makino and Yosida (1949, 1951). They are; stickiness and coalescence of chromosomes, abnormal swelling and vacuolization

of chromosomes, deformation of chromosomes into irregular bodies, disintegration of spiral chromonemata in chromosmes, lagging and non-disjunction of chromosomes, chromosome bridges, irregular chromosome distribution at anaphase, hollow metaphase, scattering or displacement of the metaphase chromosomes, the formation of the restitution-nucleus and multinulcate cells, and multipolar mitoses. In both MTK-sarcoma I and II, similar kinds of mitotic abnormalities were observed

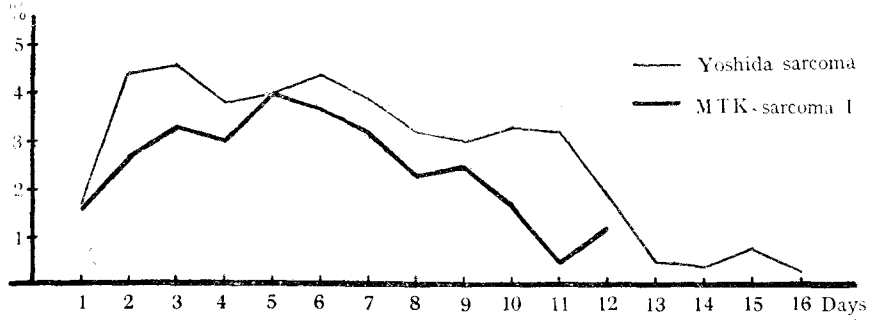

Chart. 1. Graphical representation of daily frequency of mitotic cells in a transplant generation of the MTK-sarcoma I, in comparison with that of the Yoshida sarcoma.

very frequently (cf. Kanô and Tanaka 1951). In the early generations of the successive transplantation, tumor cells of the MTK-sarcomas were variable in size as seen in Fig. 12, and chromosome bridges and lagging chromosomes at anaphase showed rather high frequency. But, with the passage of generation, no such features were observed.

6) *Chromosome number*: The chromosome numbers were observed in the MTK-sarcoma I in the same material as used for the examination of mitotic rate. The preparations were made every day through the whole life span of the host, according to the acetic gentianviolet method after Tanaka (1951).

As shown in Table 5, the chromosome numbers show a wide variation ranging from about 26 (Fig. 3) to approximately 87 (Fig. 4). The majority of the cells, that is, 71 percent of the total under observation, were found to possess 36 to 42 chromosomes, since such numbers are particularly higher in occurrence than any other. Among them, those showing the chromosome numbers, 40 or thereabouts, appeared at the highest rate (Figs. 5, 6). The number of chromosomes varies around 40, fluctuating both upward and downward in a quite gradual way. It is evident from Table 5 that the cells containing chromosome numbers lower than 40 are more frequent those having a larger number. Thus, a striking similarity appears between the Yoshida sarcoma and the MTK-sarcoma I in respect to the general accounts on the chromosome number.

Morphological analysis of chromosomes was carried out in the two new strains of the MTK-sarcoma for comparison with the Yoshida sarcoma. The results revealed that there is a striking difference in the chromosome complexes of the three strains under comparison. According to Makino (1951a, b), there is a strain of tumor cells which are primarily responsible for the formation of the tumor in the Yoshida sarcoma. These cells are characterized by having a well-balanced set of subdiploid chromosomes, 40 or thereabouts in number, which multiply dis-

Figs. 3-6. Chromosomes of the MTK-sarcoma I. Figs. 7-8. Chromosomes of the MTK-sarcoma II. The chromosome numbers in each are as follows; 26 (Fig. 3), 78 (Fig. 4), 41 (Figs. 5, 7, 8), 42 (Fig. 6). ×2200.

playing quite regular mitotic behavior. The chromosome complex of these cells is divided into two distinct groups of chromosomes: one of them consists of rod-shaped chromosomes ranging from 22 to 24 in number, while the other group comprises V- and J-shaped elements of verying sizes, from 16 to 18 in number. The results of the present observations indicated that the chromosomes of the three strains of the ascites sarcoma here concerned differ from one another in the number of V- and J-shaped elements (Figs. 5, 6, 7, 8, 17, 18, 19). This fact suggests that the MTK-sarcomas here obtained developed independently. A detailed study of

the chromosomes is in progress and the results will be published elsewhere in the near future.

7) *The behavior of the tumor cells in a transplant generation cycle of the MTK-sarcoma I* : Following the transplantation of the tumor, a large number of the tumor cells introduced into the peritoneal cavity of the new host undergo degeneration. The dividing cells are very few. The mitotic figures of tumor cells begin to appear at about 24 hours after transplantation and then show a gradual increase with time. Most of the cells undergoing division show well-balanced subdiploid chromosomes, being 40 or thereabouts in number and quite regular in general feature. On the 3rd or 4th day after transplantation, the cells with well-balanced chromosomes show very active multiplication. Towards the middle part of the life span of the tumor animal, that is, 5 or 6 days after transplantation, the cells undergoing mitosis attain the highest frequency. Towards the latter part of the life span, that is, ten or more days after transplantation, the frequency of the dividing cells with well-balanced subdiploid chromosomes decreases, while the cells in the course of degeneration proportionally increase. By the end of the life span of the host, the accumlation of the ascites reaches an enormous amount. The malignant growth of the tumor thus far progressed leads to the death of the host.

As indicated in the above description, the behavior of the tumor cells obseved in the MTK-sarcoma I through a transplant generation closely resembles that observed in the Yoshida sarcoma by Makino and Kanô (1951). Also, it is evident from the above results that the cells with well-balanced chromosomes of the ordinary configuration play an important role in the multiplication of cells for the growth of the tumor. To say it otherwise, the multiplication of tumor cells in a transplant generation is primarily attributable to the chromosomally well-balanced subdiploid cells. The details on this point are highly comparable to those observed in the Yoshida sarcoma by Makino and Kanô (1951) and Makino (1951b).

III. Remarks

Yoshida (1944, 1949) described the Yoshida sarcoma originally developed in a white rat which had been fed with O.A.T. for 3 months and then applied cutaneously with potassium arsenite solution for about 4 months. But, a question is still left open among pathologists whether the Yoshida sarcoma had been produced spontaneously or induced by O.A.T. or arsenite. According to Yoshida (1942) and Tsurusaki (1943), the lesion of the hepatic tissue was seen to recover from the cancerous condition about three months after the treatment of O.A.T. was stopped. Based on this fact, Yoshida expressed the view that potassium arsenite solution affected the induction of the Yoshida sarcoma. As set forth in the foregoing descriptions, both MTK-sarcoma I and II here under consideration were developed in the course of the hepatoma-producing-experiment with O.A.T. and D.A.B. No arsenite was used in these experiments, so that the use of O.A.T. of or D.A.B.

Table 5. Daily observations on chromosome numbers in tumor

Days \ Chrom. no.	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
1														1	1	2		1		
2										1				1	1	2				
3													1	2	1	2	3		2	2
4					1		1		2	2	2		2	1	4	3	2			
5	1					1		1	1	2	1		1	3	3	6	3	2	1	
6											1	1	3	5	7	6	4	1	2	1
7			1		1				1		2	2	1	3	3	5	4			1
8					1					2	2	1	3	1	2	8	2	2		1
9							1		2	1	2	2	1		3	1	2			
10						1	2		1		3	2	3	3	5	3	1			
11							1				1	2		1		1				1
12						1		1			1	1	1	1	2	1	1			
Total no.	1	0	1	0	3	3	5	2	7	8	15	11	16	22	32	40	22	6	5	6
%	0.45	0	0.45	0	1.35	1.35	2.25	0.90	3.15	3.60	6.76	4.96	7.21	9.91	14.41	18.01	9.91	2.70	2.25	2.70

is largely responsible for at least the development of the present tumors.

Here, it is noteworthy that in the hepatome tissue produced by the application of O.A.T. and D.A.B., there was found one cell which shows a peculiar chromosome complex just such as occurs in the tumor cell of the ascites sarcoma (Fig. 20). Since the observation was made in the preparation subjected to the squash method, the topographical position of that cell in the tissue was not clear. But it is most reasonably probable that it was not a parenchyme cell. So far as the observations have gone, such a cell as the above has never been encountered among hepatome cells. Though nothing can be stated further, this finding is important from the fact that the original cell of the ascites tumors has remained in question to the present.

IV. Summary

In the course of the hepatoma-producing-experiment by the application of azo dye in white rats, two ascites tumors which are essentially similar in nature to the Yoshida sarcoma were newly produced in two specimens. The schedule of experiments was described in this paper, with some descriptions of results of the cytological investigations of the tumor cells, by way of comparison with the Yoshida sarcoma.

The two new strains of the ascites sarcoma here established were called "MTK-sarcoma I" and "MTK-sarcoma II", respectively. Successive transplantations of the tumors have been continued from rat to rat up to the present time to 71 generations for the former and to 59 generations for the latter.

Morphological feature of tumor cells, the rate of successive transplantation,

cells of the MTK-sarcoma I through a transplant generation.

46	47	48	49	50	53	64	68	72	73	74	80	86	87	Total number of cells observed
		1			1									7
2		1												8
1				1				1			1			17
							1			1				22
1													1	28
							1							32
													1	25
						1								26
														15
														24
														7
		1												11
4	0	2	1	1	1	1	2	1	0	1	1	1	1	222
1.80	0	0,95	0.45	0.45	0.45	0.45	0.90	0.45	0	0.45	0.45	0.45	0.45	99.97

the life span of the tumor-bearing animal, the mitotic rate of tumor cells in a transplant generation, the mitotic abnormalities in tumor cells, the variation of the chromosome number in tumor cells, and the behavior of tumor cells through a whole life span were studied in the above new ascites tumors and the results were compared with those obtained in the Yoshida sarcoma. The evidence presented strongly indicated that the MTK-sarcomas here concerned closely resemble in every respect the Yoshida sarcoma.

Three strains of the ascites sarcoma show a chracteristic chromosome constitution in their tumor cells, respectively. On account of their specific chromosome complexes they are clearly distinguished from one another. The tumor cells with the specific chromosome complex, 40 or thereabouts in number, and characterized by a certain number of rod-, J- and V-shaped elements of varying sizes, multiply with a regular behavior. Observations through a transplant generation revealed that these strain cells primarily contribute to the growth of the tumor.

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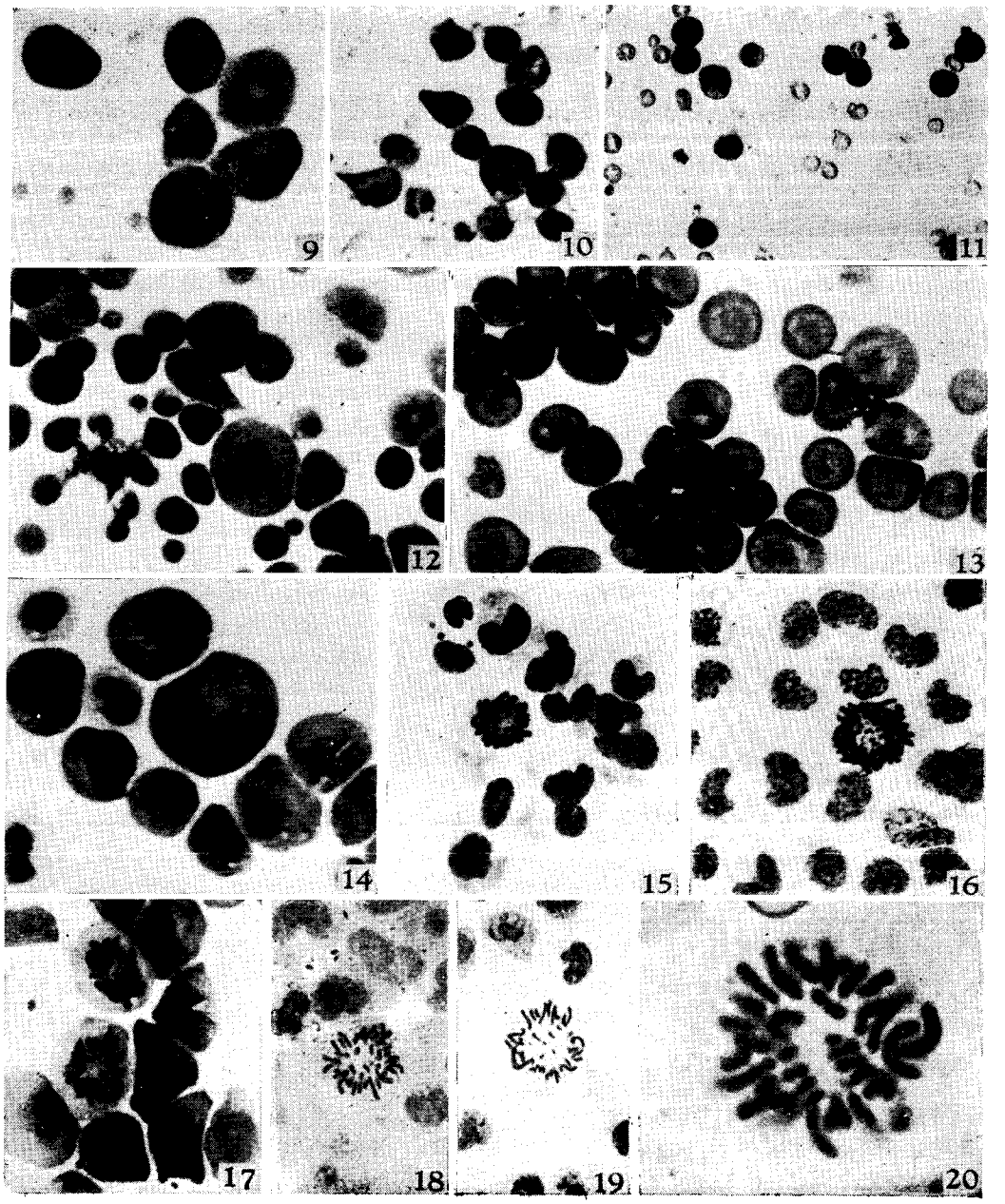
Explanation of plate XIII

Figs. 9-11. The tumor cells containing hightly basophilic cytoplasm, developing in the peritoneal cavity of the original animal of the MTK-carcoma I. There are visible a remarkable nucleolus in each nucleus. In Fig. 11, tumor cells are shown together with erythrocytes. (9 ; ×600. 10-11 ; ×400). **Figs. 12-14.** Tumor cells of varying sizes, from the MTK-sarcoma I. **12,** tumor cells from the 1st transplant generation of MTK-sarcoma I. (×400). **13,** tumor cells from the 2nd transplant generation of the same. (×400). **14,** tumor cells from the 3rd transplant generation of same. (×600). **Figs. 15-17.** The tumor cells of the MTK-sarcoma II, containing dividing cells. **15,** tumor cells from the original animal of the MTK-sarcoma II. (×600). **16.-17,** tumor cells from the 1st transplant generation of the same. (×600). **Figs. 18-19.** Metaphas figures of well-balanced tumor cells showing approximately 40 chromosomes, obtained from the MTK-sarcoma I. (×600). **18,** from the material at 7 days after transplantation in the 1st transplant generation of MTK-sarcoma I. **19,** from the material at 7 days after transplantation in the 8th transplant generation of the same. **Fig. 20.** Metaphase plate of a hepatoma cell possessing the chromosome complex similar to that found in the ascites tumor. (×1600). **Figs. 9-14 and 17,** from Giemsa preparations. **Figs. 15-16 and 18-20,** from acetic gentianviolet-preparations.

(*Photo by Prof. Makino*)



Tanaka, T. & K. Kanó: General Characters of the New Ascites Tumors.