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Cytological Studies on Cancer

I. Morphological and Statistical Observations on the Abnormal Mitosis in Tumor Cells of the Yoshida Sarcoma Through a Transplant Generation¹⁾

By

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(With 55 Figures in 8 Plates)

Introduction

Nowadays, the cytology of cancer, especially in the field of the chromosome study, from both morphological and physiological standpoints, has attracted particular concern of many investigators of tumor pathology concomitant with biological interest, primarily because the fundamental ground of cancer research may be built up with cytological phenomena. Heretofore, a considerable amount of work has been done in the field of cancer cytology; a high mitotic rate and a high frequency of abnormal division have been described as almost universal phenomena in tumors. The papers published by Goldschmidt & Fischer (1929), Lewis & Lockwood (1929), Kemp (1930), Ludford (1930), Levine (1931), Ishibasi et al (1931), Alexenko & Natansohn (1932), Amano & Ando (1940), Biesel et al (1942, 1945), Hauschka (1945-1948), and Casabona (1948) are important reports of cytological studies carried out with the tumors of rats and mice. Beginning early with the work of Hansemann published in 1891, Galeotti (1893), Heiberg & Kemp (1929), Levine (1930), Picon (1930), Winiwarter & Oguma (1930), Ohta (1933), Papanicolaou (1942, 1943), Barigozzi (1947), Barigozzi & Dellepiane (1947), Koller (1947), Barigozzi & Grattarola (1950), Polli (1950), Timonen (1950), Timonen & Therma (1950) and Therma & Timonen (1950) have made excellent contributions to the cytology of human cancer. A review of older literature has been given by Politzer (1935).

1) Contribution No. 254 from the Zoological Institute, Faculty of Science, Hokkaido University, Sapporo, Japan.

In reviewing the previous studies, it is evident that the attention of most of these authors was attracted to the variation of the chromosome number, various mitotic abnormalities and some related phenomena occurring in tumor cells. It is important and desirable to make inquiry into the behavior of tumor cells or the karyological changes occurring in them during the course of the growth of the tumor.

Unfortunately, for the purpose of cytological study the stroma-forming solid tumors, such as those removed from human bodies or those naturally or experimentally developed in rats and mice, long used as material by previous workers, have proved unfavourable. In addition, many technical difficulties associated with the classical paraffin method prevent the progress of study in this field to a considerable extent. Thus our knowledge on the cytology of cancer is much less satisfactory than is that in any other fields of cancer research. While referring to reports recently published by T. Yshida and his co-workers (1944, 1949) concerning the so-called Yoshida sarcoma, the authors received an impression that the latter sarcoma provides a number of favorable conditions for the cytological investigation. The Yoshida sarcoma, an ascites tumor, grows in the peritoneal cavity of white rats in a form of fluid tumor and is capable of successive transplantations. It allows of microscopical tracing of the behaviour and condition of tumor cells through every developmental stage of the tumor in a transplant generation, with a simple smear technique. A series of studies to be published hereafter will be carried out mainly on this sarcoma. Previously, some works on cytology of this tumor have been published, and fragmental evidences have been reported (Yosida & Makino 1949, Yosida 1948, 1949, a, b, Atsumi 1949, Sato 1950, Hirono 1951, Makino 1951).

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General characteristics of the Yoshida sarcoma

General accounts of the Yoshida sarcoma were rendered in detail by Yoshida (1944, 1949) who had established this tumor as a strain. But, it may be useful to make here some mention of the characteristic feature of this tumor. According to Yoshida (1949), the Yoshida sarcoma originally developed in an albino rat which had been fed with o-amidoazotoluene for 3 months and then applied cutaneously with potassium arsenite solution for about 4 months. The tumor ascites is of milky appearance and 1 cc of it contains approximately one million

tumor cells in a state of suspension. The transplantation can readily be made by injection of a small amount of tumor ascites into the peritoneal cavity of a new rat, with the aid of a sharply pointed glass pipette (Yoshida 1949). By this simple method, successive transmissions of the tumor have been made from rat to rat. After transplantation the tumor cells rapidly proliferate chiefly in the abdominal cavity of the new host, with a remarkable increase of ascites. The tumor-bearing animal dies in 12 days on the average.

Viewed from the cytological standpoint, the Yoshida sarcoma has many advantages for study as follows:

- 1) The tumor cells are surprisingly larger in size than any blood cells or other somatic cells occurring in the peritoneal cavity. This renders microscopical observation extremely easy.
- 2) The developmental condition of tumor cells in the growth of the tumor, and the process of their division can easily be observed in a single tumor animal at any time desired, or can be traced successively through the duration covering the time just after transplantation until the death of the tumor animal.
- 3) The simple and rapid smear method by the application of Giemsa's stain or acetocarmine allows close study for cytological purpose.

Technique

As mentioned above, the Yoshida sarcoma proved to be a favourable material for the cytological study in that the material for examination is available obtained at any time when wanted with a fine pipette without dissecting the animal, and further that the smear preparations are available for the chromosome investigation with satisfactory results. In the following-described investigations, both the Giemsa's azur-eosin-methylenblue solution for blood smear and the temporary smear technique with acetocarmine were employed. The procedure of Giemsa's method is thus; a small amount of ascites containing tumor cells is smeared on a slide from a droplet of the ascites which was obtained by inserting the glass pipette into the abdomen of the tumor animal, and fixed with absolute menthol for a few minutes. The slide is then allowed to dry and stained with Giemsa's solution for 40-60 minutes. After staining the preparation is washed with distilled water until properly differentiated and then blotted dry. In the acetocarmine smear, the fresh ascites smeared on a slide is directly covered with acetocarmine. It is desirable to leave the smear for about 20 minutes before applying the cover-slip. To seal the cover-slip, a new sealing medium is to be used (Makino & Nishimura 1952). The substitution either of acetic orcein as recommended by La Cour (1941) or of acetic gentianviolet as advised by Tanaka (1951) for acetocarmine, is likewise available for study with satisfactory results respectively.

The Giemsa-preparations are useful for the diagnostic observation of tumor cells, since by this method the criteria for morphological and physiological changes of tumor cells are to be obtained. But, this method is disadvantageous for the research of chromosomes; the cells become flattened by drying up the smear material

and this causes the shrinkage of cells showing artificial cohesion and irregular deformation of chromosomes. However, the acetocarmine smear technique can be employed with several advantages in the chromosome study, of which the most important are its simplicity, speed of procedure, and the results in both preservation and staining of chromosomes. Both cells and chromosomes are strikingly free from shrinking. The chromosomes prepared with this technique are observed with a well-defined, sharp outline and extreme clearness of appearance. Acetocarmine can be successfully substituted for acetic orcein or acetic gentianviolet as noted above.

The albino rats (*Rattus norvegicus*) which are derivatives of the strain from the Wistar Institute and have been bred pure in our laboratory were exclusively employed as host animals for the transmission of the tumor.

Observations

1. Descriptions of mitotic abnormalities in tumor cells

The tumor cells are very prominent by reason of being larger in size than any other cells observable in the peritoneal cavity. The nuclei are characterized by oval or kidney shape, or sometimes bilobed appearance, and locate on one side of the cell (Fig. 35). Conspicuous bodies, generally two or more in number, are visible within the nuclear body. Probably they are nucleoli because they are basophilic and negative to Feulgen reaction. In the wider space of the cell, distinct azurophilic granules make their appearance in cytoplasm arranging in a remarkable rosette form, when stained with Giemsa's stain.

It has frequently been reported by many investigators of tumor cytology that a high mitotic rate and a high frequency of abnormal division are almost universal phenomena in tumors (*cf.* Politzer 1935). The same can be said as to the Yoshida sarcoma here concerned. The smears prepared at any time from the ascites of the tumor animal exhibit a number of dividing tumor cells as well as various types of abnormal mitosis. A considerable daily variation occurs in the rate of cell division after transplantation. During about the first day after the transplantation of the tumor, the majority of the tumor cells undergo degeneration in the peritoneal cavity of the new host. The dividing cells are few in number. Afterward the division rate of cells progressively increases with time, and by the 5th or 6th day after transplantation, that is, in the middle part of life span of the tumor animal, the division of tumor cells is found at the highest frequency. Towards the latter part of the life span, that is, ten or more days after transplantation, the frequency of dividing cells decreases, while the cells in the course of degeneration proportionally increase. Throughout the course of the growth of the tumor, abnormal mitoses were observable at a high frequency showing a great variation in details of type.

In view of the fact that mitotic abnormalities are almost of common occurrence in tumors generally, it seems necessary to describe and illustrate in detail

these mitotic abnormalities in order to analyse the causes underlying them. Of the mitotic abnormalities¹⁾ occurring in the Yoshida sarcoma, the following types are remarkable :

Stickiness of the chromosomes is common, especially observed in the cells which are in disintegration (Figs. 1, 36). The chromosomes exhibit stickiness in more or less degree and fuse at points of contact specially at the ends. It is undoubtedly these sticky chromosomes that form chromatic bridges at anaphase stretching between the poles (Fig. 2), and lagging behind the regularly separating chromosomes (Fig. 40). Externally the stickiness seems to be dependent on cohesion of matrical substance of the chromosomes. Sometimes it happens that there is a loss of chromosome material in one of the daughter cells, while excess of them – namely, a partial doubling of chromosomes – in the other, occurs as a result of incomplete separation caused by the stickiness of chromosomes. Also, the fragmentation of the chromosomes seems, in most cases, to take place in close connection with the bridge-formation of sticky chromosomes. The size of fragments shows a great variation. Also, some chromosomes may be caught between the bridges, in which case they will be excluded from the nucleus. Thus, irregular and incomplete separation of chromosomes results from stickiness, and this gives rise to cells with externally variable chromosome contents. Extreme stickiness completely prevents the separation of the telophase chromosomes and this makes the cell division incomplete. In some cases, the daughter nuclei, after full division, remain associated by connecting chromosomal bridges (Fig. 43). The coalescence or irregular agglutination of chromosomes, a feature common in degenerating cells, is probably attributable to the extreme case of stickiness (Fig. 37). The abnormal swelling of chromosomes is also one of the noticeable changes here observed. When the swelling advances, the deformation of chromosomes into unusually rounded bodies results (Figs. 3, 38). The breaking down of the spiral chromonemata within chromosomes is also commonly observed (Figs. 4, 5, 39). Obviously, such a cell undergoes degeneration at metaphase without advancing further. These abnormalities have been considered to be a result of the abnormal metabolic change within the chromosomes.

Atypical arrangement of chromosomes at metaphase is of rather common occurrence in tumor cells. The chromosomes may be irregularly scattered about on the equatorial plane instead of taking the regular radial arrangement, or clumped together occupying an eccentric area (Fig. 6). Often the metaphase displays some chromosomes lying extruded outside the nuclear plate (Figs. 8, 42). The lagging chromosomes found at anaphase seem mostly to result from such extruded elements. Sometimes the chromosomes lie more contracted than usual.

1) Preliminary notes on this point were published by Yosida (1948), Makino and Yosida (1949), and Makino (1951), but in the present report more detailed accounts recently obtained will be given.

The occurrence of hollow metaphase plates in which the centre is without chromosomes is likewise a remarkable abnormal feature observable in tumor cells (Fig. 7). There are some intermediate configurations between normal metaphase plates and completely hollow plates. Evidently, hollowness is governed neither by a small number of chromosomes nor by the sticky nature of the chromosomes. It is obvious that these abnormalities are largely correlated with the deficiency of the spindle mechanism.

Lagging of the chromosomes during anaphase is a phenomenon of relatively common occurrence in tumor cells (Figs. 9, 10, 41). The laggards being 1 or 2 in number are most usual, but there are cases which show 5, 6 or even 10 laggards. The laggards may include the chromosomes undergoing non-disjunction, as well as those lying off the metaphase plate. The size of laggards shows also a wide variation. The lagging elements are either included in the separating chromosomes or excluded outside the daughter nuclei. The latter case is very common; it follows either the elimination of laggards or the formation of the micro-nucleus. It is a rather common phenomenon that the multipolar divisions, at least in anaphase, contain lagging chromosomes. Obviously, lagging of chromosomes during anaphase results in nuclei in which some chromosomes are absent, or in which extra, additional chromosomes are included. Unequal distribution of chromosomes at anaphase, or a migration of chromosomes in different numbers to poles, is also a pronounced feature (Figs. 11, 12). Though this phenomenon is often observed in giant cells undergoing polypolar division, it by no means always concerns multipolarity or polyploidy. It can be abundantly seen in the bipolar mitosis. Unequal distribution of chromosomes during anaphase is probably due to the failure of synchronization in separation of chromosomes, and it is usually brought about in close association with partial breakdown of the spindle mechanism.

The formation of the restitution-nucleus is indicated by the occurrence of polyploid cells with well-balanced chromosomes which show the regular metaphase arrangement and the normal bipolar spindle. Rather frequently, the restitution-nucleus assumes a bilobed appearance with two lobes of similar or dissimilar outline and remains associated by a narrow connection (Figs. 13, 14, 43, 44).

The multipolar mitosis occurs in a rather large proportion of cases as a remarkable feature of this sarcoma (Figs. 15, 16, 17, 19, 45, 46, 47, 48). It is seen either in actively dividing subdiploid or hypoploid cells or in giant cells. Although the giant cells show usually several poles in division, multipolarity is by no means always restricted to polyploidy. One can observe very small cells which show tripolar spindle, or also polyploid metaphase plates of a normal bipolar nature. The most usual type of multipolar division is tripolar division (Figs. 15, 16, 22, 46); observations showed that 73 percent in the total of the multipolar mitosis were tripolar. Tripolar divisions are more frequent in subdiploid cells than in hyperploid cells. The tripolar division results in different numbers of chromosomes at

three poles. For example, Figure 15 shows a tripolar division which had taken place in a subdiploid cell; in one of poles are nearly $2n$ chromosomes, while the other two poles show about 25 and 11 elements, respectively (Fig. 15). Example of quadripolar mitosis given in Figure 17. Pentapolar as well as hexapolar mitoses are rather rare. In Figure 48 an octopolar anaphase is depicted; it is likewise rarely found. Multipolar divisions of the giant cell forming many spindles are not of uncommon occurrence; generally, they are too complicated to be analysed in detail (Fig. 18). Most of these multipolar divisions contain lagging chromosomes together with chromosome bridges stretching between poles. These divisions result in the irregular migration of chromosomes in different numbers to poles at anaphase. The multipolar division takes place not only in polyploid cells, but also in multinucleate cells. But, the latter seems to be of rather common occurrence. The multipolar metaphase plates generally show abnormal orientation of the metaphase chromosomes. Sometimes the chromosomes lie in two or more layers at metaphase (Figs. 19, 20, 21, 45, 47). These findings suggest that the multipolar spindles mostly originate from the multinucleate cells. Generally speaking, the multipolar divisions result in the production of either a multinucleate cell with variable numbers of chromosomes, or a many lobed restitution-nucleus. For example, in the tripolar division, there is a formation of not only three cells with different number of chromosomes (Figs. 15, 16), but also two cells one of which is a bi-nucleate cell and the other a mono-nucleate cell (Fig. 22). If the tripolar division takes place in the subdiploid cell, a cell with a reduced number of chromosomes would be produced. Figure 23 shows a tetrapolar division resulting in the formation of two cells, one of which receives three nuclei and the other a single nucleus. Figure 51 illustrates a very huge giant cell in which a many-lobed restitution-nucleus was formed. In the giant multipolar metaphases the chromosomes very often show the beginning signs of disintegration forming amorphous chromosome masses (Figs. 49, 50). It seems highly probable that when the cell has reached a certain size it becomes inviable.

Referring to the above data, it may be understood that for the change of the nuclear contents the multipolar divisions take significant part as direct or indirect causes, giving rise to the cells with the extremely variable chromosome numbers. Thus, the abnormalities of the cell progressively increase due to a change in the number, structure and behavior of chromosomes from cell to cell with the progress of division. In the next step, the normal cellular activity stops probably due to the deficient nuclei which have resulted from abnormal mitoses. Finally the cells become inviable. Generally the percentage of multipolar metaphases in the total is considerably higher than that of multipolar anaphases. For instance, out of 73 percent of the tripolar division, 64 percent was found to be in the metaphase stage. This seems to suggest that most multipolar metaphases never reach the anaphase stage. A comparable feature was reported in human cancers (Timonen

and Therman 1950).

A further pronounced and common feature is the occurrence of multinucleate cells. The bi-nucleate cell is most usual and very frequent in appearance. The two nuclei contained in such a cell are approximately similar in size and shape in many cases (Figs. 24, 53), with a few exceptions in which they are of dissimilar size (Fig. 54). Though not so abundant, tri-, quadri-, penta-, and hexa-nucleate cells were likewise met with (Figs. 25, 26, 52, 53). Occasionally, so-called giant cells were observed which are characterized by the presence of some ten or more nuclei containing a considerable amount of cytoplasm. It is a general feature that the nuclei contained in these multi-nucleate cells are strikingly different from one another in both size and shape, and sometimes even in stage. In many cases, the nuclei contained in a cell undergo fairly synchronous division each showing the same phase of division (Figs. 27, 55), but in a few cases the nuclei did not show agreement in the stages of division (Fig. 28). The bi-nucleate as well as tri-nucleate cells assumed such a configuration. Commonly, the multi-nucleate cells are closely associated with the development of a multipolar spindle. In the multinucleate cells the chromosome plates are usually formed with an abnormal orientation (Figs. 20, 21).

The daily frequency¹⁾ of the multinucleate cell was calculated through the life span (16 days) of a tumor-bearing rat on the basis of 2000 cells per day (32000 cells in total). The results are as follows; the bi-nucleate cells occur in 30.1%, the tri-nucleate cells in 1.3%, the quadri-nucleate cells in 0.3%, the penta- and hexa-nucleate cells in 0.1%, respectively. Through a transplant generation the frequency of the bi-nucleate cell was found relatively high in the early part of the life span, whereas multi-nucleate cells containing three or more nuclei tended to increase in number from the middle part to the latter part.

The numerical change of the chromosomes has been described by many previous investigators in various human cancers as well as in experimental tumors of rats and mice as an almost universal phenomenon in tumor cells (cf. Makino's list 1951). Various deviations of the chromosome number from the normal one (42) were encountered in the Yoshida sarcoma too, as a remarkable abnormality of common occurrence. The counting of the chromosome number made in a number of metaphase plates indicated that the chromosomes showed a wide numerical variation ranging from about 20 to more or less than 85. Hence, the lowest number so far obtained in this sarcoma approximates 20, while the highest one appears as about 85 (Figs. 29, 30), except in the giant cell. Between these two extremities, the numbers exhibit a rather gradational fluctuation. Here important and noticeable is the fact that cells carrying 40 to 41 chromosomes (Figs. 31, 32, 33), which are nearly approximate to the normal number of the host animal (*Rattus norvegicus*;

1) The data in this respect were furnished through the courtesy of Miss K. Kanô.

$2n=42$, Fig. 34), were most frequent in occurrence. These cells appear to show a definite mitotic rate through the whole period of the life span of the tumor animal. Around 40-41, the chromosome numbers fluctuate both upward and downward in a quite gradual way. No true polyploidy seems to occur, but the cells close to triploid or tetraploid numbers were rarely found (Fig. 30). The giant cells with a very high number of chromosomes such as about 200 or more make also occasional appearance. Exact counting of chromosomes was impossible in these cells, since the metaphase orientation of chromosomes is mostly abnormal showing commonly the multipolar spindle (Fig. 18).

As above mentioned, the abnormalities concern the behavior of the chromosomes, or the spindle, or both. Undoubtedly, most of them lead to degeneration and death of the cells. It needs to be emphasized that cells which are undergoing abnormal mitosis cannot continue active multiplication and therefore they contribute little to the growth of the tumor. Here, particular attention should be paid to the fact that the cells having the subdiploid chromosome number (40 or thereabouts) are always very frequent showing a definite mitotic rate (Figs. 31, 32, 33), and further that they are quite regular in the behaviour of chromosomes in the course of division. It is highly probable that cells containing the subdiploid chromosomes play a significant part in the growth of the tumor because of their regular mitotic activity. On this point, details will be reported in another paper of this series of studies.

By way of summary the mitotic abnormalities described above are recognized as remarkable cytological phenomena in the Yoshida sarcoma. Broadly considered, these mitotic abnormalities can be arranged into two main categories: namely *i*) abnormalities involving structural alterations in the chromosomes, and *ii*) those attributable to accelerated, suppressed, delayed or incomplete spindle formation. To the former category may belong: stickiness and coalescence of chromosomes, abnormal swelling or vacuolization of chromosomes, deformation of chromosomes into unusual irregular bodies, and disintegration of spiral chromonemata in chromosomes. Probably these abnormalities are attributed to a change or changes in the organization of chromosomes during mitosis, for instance to an excess of deficiently polymerized nucleic acid as emphasized by Koller (1947). Into the latter category fall these abnormalities: lagging of chromosomes at anaphase, non-disjunction of chromosomes, chromosome bridges at anaphase and telophase, irregular chromosome distribution at anaphase, hollow metaphase, scattering or displacement from normal arrangement of chromosomes at metaphase, some chromosomes lying off the equatorial plate, abnormal orientation of metaphase chromosomes, the formation of the restitution-nucleus and multinucleate cells, and multipolar mitoses. Obviously, almost all these abnormalities contribute as direct or indirect causes for the variation of chromosome numbers, resulting finally in highly complicated duplication of chromosomes as seen in the giant cells.

The abnormalities of these categories can almost all be brought about in normal cells by various therapeutic agencies or by experiments in plants and animals, for instance by subjecting cells to high or low temperatures, by treating them with chemicals or some hypotonic or hypertonic media, or by exposing them to X-rays or γ -rays. The similarities between the abnormalities which occur in cancer and those induced by these artificial means are very striking. This problem will be touched upon again elsewhere.

2. The frequency of mitotic abnormalities

In the foregoing section, a number of mitotic abnormalities occurring in the Yoshida sarcoma were described with respect to their general morphology. In

Table 1. Total frequency of mitotic abnormalities in tumor cells observed in a tumor rat through 9 days of its life.

Type of abnormalities	Frequency (%)
Division type	36.8
Aberrant type :	25.8
<i>Hypo- & hyperploid</i>	1.3
<i>Multinucleated & multipolar cells</i>	3.8
<i>Irregular distribution of chroms. at anaphase ; lagging and non-disjunction</i>	6.8
<i>Displacement and abnormal orientation of metaphase chroms. ; hollow metaphase</i>	4.2
<i>Deformation of chroms.</i>	0.4
<i>Slight stickiness of chroms. ; chromosome bridges</i>	2.9
Disintegration type	37.2
Total number of cells observ.	236
%	99.8

Table 2. Daily frequency of the mitotic abnormalities. A, from the same

Indiv.	Type	Life days	1	2	3	4	5	6
			%					
A	Division type	53.6	31.2	46.8	20.8	42.8	51.6	
	Aberrant type	26.8	28.1	19.1	33.3	32.1	25.8	
	Disintegration type	19.5	40.6	34.0	45.8	25.1	22.5	
B	Division type	15.5	32.5	29.2	33.4	—	23.4	
	Aberrant type	62.0	54.9	57.5	60.0	—	43.3	
	Disintegration type	22.5	12.6	13.3	6.6	—	33.3	
C	Division type	—	12.5	20.0	20.0	—	30.0	
	Aberrant type	—	56.2	67.0	30.0	—	56.7	
	Disintegration type	—	31.9	13.0	50.0	—	13.3	

this section are presented the results of observations carried out on the frequency of mitotic abnormalities in tumor cells studied through the whole life span of the tumor-bearing animal. A droplet of tumor ascites was obtained once a day from a certain tumor animal, and smears were made for microscopical examination according to Giemsa's technique. Based on these preparations daily observations were made on the frequency of abnormal mitosis in tumor cells. The data were obtained from the dividing cells alone.

For the sake of convenience in description the tumor cells were divided into the following three types :

i) *Division type* ; this includes the cells which seem to undergo regular division with active mitotic behavior, showing the subdiploid chromosome numbers, 40 or thereabouts.

ii) *Aberrant type* ; to this type belong the cells showing many abnormalities, as described in the foregoing section.

iii) *Disintegration type* : in this group are included the cells where disintegration processes are seen in progress. They cover abnormalities such as coalescence or clumping together of chromosomes, abnormal vacuolization or swelling of chromosomes, breaking down of spiral chromonemata within chromosomes and giant cells with amorphous masses of chromosomes.

Firstly, the total frequency of abnormal mitosis occurring in tumor cells was observed in a certain tumor rat through its whole life span (9 days in this case), based on the daily material. The numbers of cells thus obtained were calculated in percentage on the basis of the total number of observed cells. The results were arranged in the three types above mentioned, as given in Table 1.

Next, the daily frequency of the mitotic abnormalities in tumor cells was observed based on the same material as used in Table 1. The results were given in Table 2, together with two other examples obtained from another material. As indicated in Table 2, the daily frequencies of the mitotic abnormalities furnish

material as Table 1. B and C, based on the material from two different individuals.

7	8	9	10	11	12	13	14	15	16
0 20.6 79.3	0 25.0 75.0								
21.5 75.2 3.3	20.0 65.8 14.2	6.6 70.1 23.3	— — —	3.4 38.0 58.6	0 20.0 80.0	16.0 28.0 56.0			
13.3 66.7 20.0	10.7 55.6 33.3	11.1 55.6 33.3	16.6 53.4 30.0	17.2 65.6 17.2	6.9 55.5 37.6	3.7 59.3 37.0	13.3 56.7 30.0	7.1 50.0 42.9	3.3 51.5 45.0

an interesting and significant feature regarding the growth of the tumor. The frequency of cells belonging to the division type gradually increases from the early part of the life span of the tumor animal toward the middle part, but decreases passing to the latter part. Cells of the aberrant type, however, exhibit the highest frequency of all during through the middle part of the life span betraying a decreasing tendency toward the latter part; those of the disintegration type are exclusively high in frequency in the latter part along with their considerable occurrence in the early part.

Based on the above data, the cycle of tumor cells in the development of the tumor can be described as follows: the tumor ascites inoculated in the peritoneal cavity of the new host probably contain cells of three types. The cells of the division type multiply through active mitosis and increase gradually in number during the period from the early part of the life span to the middle part together with the gradual increase of ascites, while those of the aberrant type tend to decrease during the early days, due to degeneration through abnormal mitosis. At the same time, the increase of cells of the disintegration type is brought about during the corresponding period. At the middle part of the life span when the most active malignant growth of the tumor is attained, the mitotic cells in the ascites are mostly of the division type. Then, the cells of the division type decrease in number with the passage of time. This decrease of cells of the division type corresponds to the increase of aberrant cells as well as of disintegrating cells. Most probably, this is due to the transition of cells of the division type into those of the aberrant or disintegration types. Obviously, the latter are unable to continue division. Hence, therefore, in the latter part of the life span, the majority of dividing cells are abnormal, and the disintegrating cells appear at the highest frequency. In the latter part, however, most cells of the division type remain inactive persisting in the resting condition. In reality, among degenerating cells, there occur a large number of resting cells which are characterized by small amount of cytoplasm and basophilic nuclei. The evidence presented suggests that the proliferation of tumor cells in the host by successive transplantations may primarily be attributable to the multiplication of cells of the division type, contributing to the growth of the tumor. This is one of the most important items pertaining to the mechanism of the malignant growth of the tumor, and therefore demands further confirmation of the fact. It would be premature to attempt to reach final conclusions as to the phenomenon from the results of the present investigation.

Before finishing, it may not be out of place to state here the hydration and dehydration theory on the cause of abnormal mitosis postulated by Kuwada (1937) and his followers. According to Kuwada, a change of water relation in the cell plays an important role in the production of mitotic abnormalities. Based on this theory, some mention will be made on the mitotic abnormalities occurring in the tumor. It has been shown that most of the mitotic abnormalities of common occur-

rence in nature and in experiments are regarded as being connected largely with the disturbance in normal water relation in the cell (Kuwada 1937, Shinke 1939, Sigenaga 1949, Nakamura and Makino 1950, Kanô 1951). The similarities between the abnormalities occurring in cancer and those induced by treating the cells with hypotonic or hypertonic media are very striking (Shinke 1939, Sigenaga 1949, Kanô 1951). As described in the foregoing pages, in this sarcoma tumor cells of the division type continue an active multiplication through a regular course of mitosis during the time when the peritoneal fluid of the host is still in probably undiseased condition. But, by the time when the peritoneal condition becomes unfavourable in the middle part of the life span due probably to the remarkable accumulation of the tumor ascites, cells of both aberrant and disintegration types increase rapidly with time. The accumulation of the tumor ascites, which consists in the greater part of degeneration products of tumor cells, probably brings about the change in viscosity of ascites. This change in the viscosity of ascites, in which tumor cells occur in a form of suspension, would disturb the water relation in tumor cells, if not directly, then in more or less degree as response to the osmotic pressure in the surrounding medium. This may be followed by changes of normal spindle mechanism or by the structural alteration of chromosomes due to dehydration or hydration processes, thus leading to various mitotic abnormalities. In addition, the substance produced by degeneration of tumor cells would contain something which acts as a mitotic poison for tumor cells. The sudden increase of abnormal mitotic figures of tumor cells in the latter part of the life span is very suggestive of this probability. We will have an opportunity in the near future to touch again this problem on the basis of the data accumulated in various experiments.

Summary

The Yoshida sarcoma here used as material is an ascites-sarcoma which grows in the peritoneal cavity of white rats (*Rattus norvegicus*) in a form of malignant fluid tumor. Successive transmission is carried out with ease by intraperitoneal inoculation of a droplet of the ascites containing tumor cells; the tumor cells rapidly multiply in the new host with a remarkable production of ascites, leading to the death of the tumor-bearing animal in 12 days on an average. The Yoshida sarcoma possesses many advantages for cytological investigation.

Various types of mitotic abnormalities occurring in the Yoshida sarcoma and the frequency of their occurrence have been described in this paper. On the basis of the daily frequencies of mitotic abnormalities observed through a transplant generation, the view was expressed that tumor cells which are undergoing regular mitosis with well-balanced subdiploid chromosomes primarily contribute to the growth of the tumor.

From the fact that striking similarities exist between the abnormalities occurring in the tumor and those induced by treating the cells with various chemicals,

or by hypotonic or hypertonic media, a tentative statement was made that a change in normal water distribution in the cell may be regarded as a cause of the mitotic abnormalities occurring in the tumor.

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Explanation of plate VII

All are camera-lucida drawings of tumor cells, $\times 1300$.

Fig. 1, stickiness of chromosomes. **Fig. 2**, chromosome bridges at anaphase. **Fig. 3**, deformation of chromosomes into rounded bodies. **Figs. 4-5**, breaking down of spiral chromonemata within chromosomes. **Fig. 6**, irregularly scattering of chromosomes in the metaphase plate. **Fig. 7**, hollow metaphase. **Fig. 8** showing some chromosomes lying extruded outside the equatorial plate. **Figs. 9-10**, lagging chromosomes at anaphase. **Figs. 11-12**, unequal distribution of chromosomes at anaphase. **Figs. 13-14**, formation of restitution-nucleus. **Figs. 15-16**, tripolar mitoses. **Fig. 17**, quadripolar mitosis.

Explanation of plate VIII

All are camera-lucida drawings of tumor cells, $\times 1300$.

Fig. 18, multipolar divisions in a giant cell. **Figs. 19-20**, tripolar mitoses, metaphase. **Fig. 21**, quadripolar mitosis, metaphase. Two equatoroal plates lie at about right angle. **Fig. 22**, telophase of a tripolar mitosis, forming a binucleate cell. **Fig. 23**, telophase of a quadripolar mitosis forming a tri-nucleate cell. **Fig. 24**, bi-nucleate cell. **Fig. 25**, trinucleate cell. **Fig. 26**, tetra-nucleate cell. **Fig. 27**, two metaphase plates in a bi-nucleate cell. **Fig. 28**, showing asynchronous division in a bi-nucleate cell. **Fig. 29**, metaphase plate showing 22 chromosomes. **Fig. 30**, metaphase plate showing 80 chromosomes (subtetraploid). **Figs. 31-33**, metaphase plates showing well-balanced subdiploid chromosomes; 41, 42 and 41 elements in each. **Fig. 34**, metaphase plate of a blood cell showing 42 chromosomes.

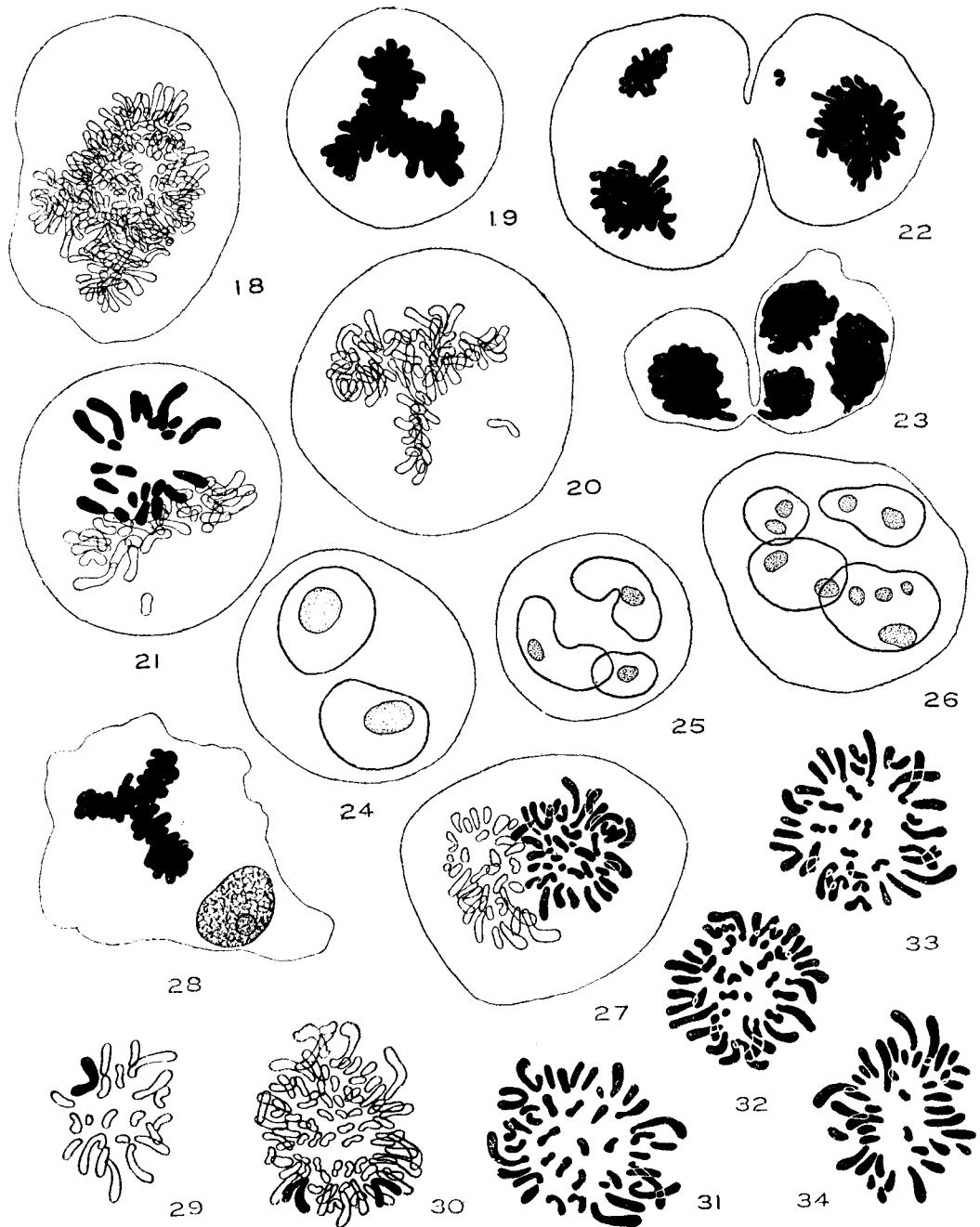
Explanation of plate IX

All are photomicrographs of tumor cells.

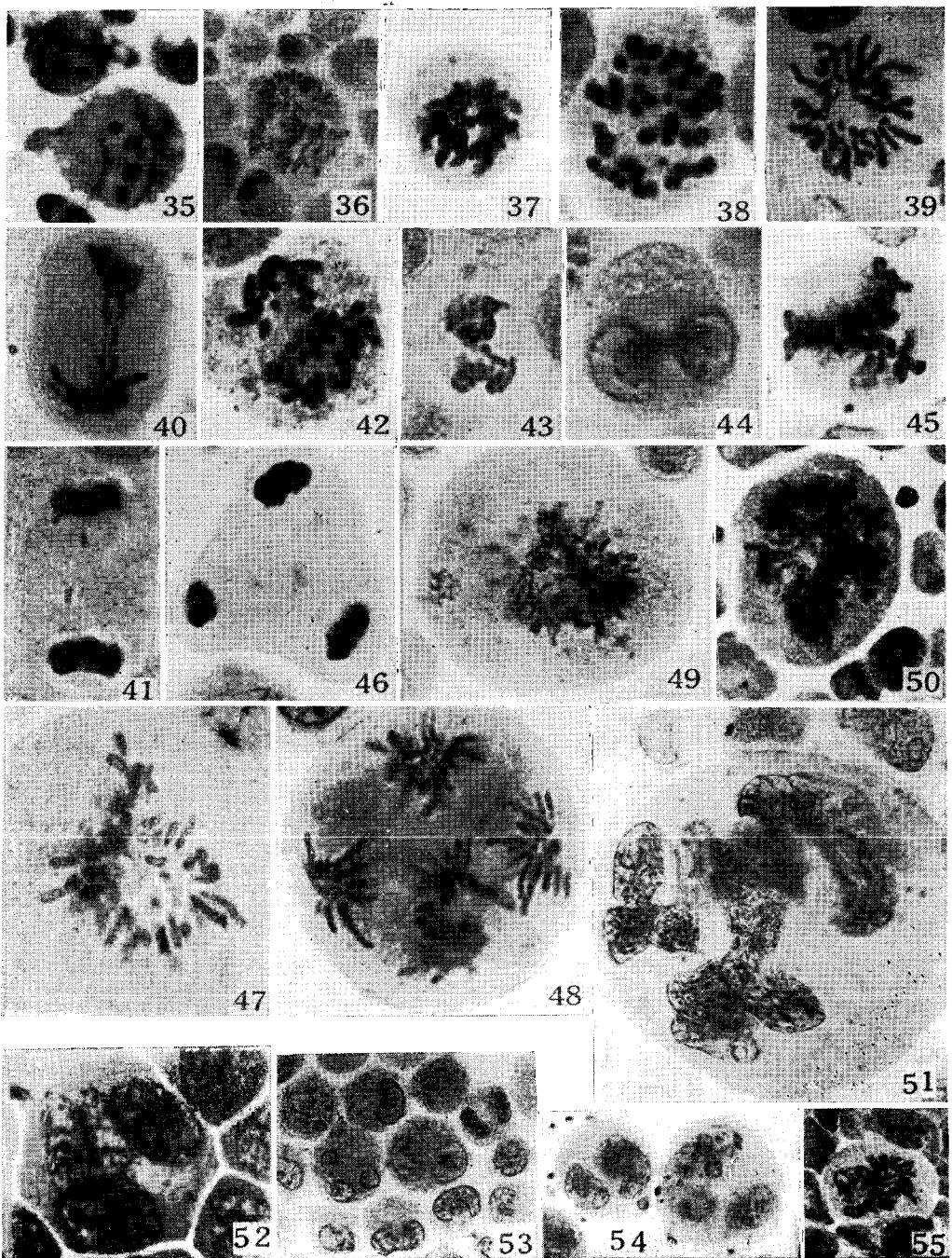
Fig. 35, tumor cells at resting stage, showing remarkable nucleoli in nuclei. **Fig. 36**, stickiness of chromosomes. **Fig. 37**, coalescence of chromosomes. **Fig. 38**, deformation of chromosomes into rounded form. **Fig. 39**, breaking down of spiral chromonemata. **Fig. 40**, chromosome bridges at anaphase. **Fig. 41**, lagging of chromosomes at anaphase. **Fig. 42**, showing some chromosomes extruded outside the equatorial plate. **Fig. 43**, formation of restitution-nucleus. **Fig. 44**, two nuclei with narrow connection. **Fig. 45**, tripolar metaphase. **Fig. 46**, tripolar telophase. **Fig. 47**, tetrapolar metaphase. **Fig. 48**, octopolar anaphase. **Figs. 49-50**, giant cells showing amorphous masses of chromosomes. **Fig. 51**, giant cell containing lobulated nuclei. **Fig. 52**, tri-nucleate cell. **Fig. 53**, showing bi-nucleate cells and tri-nucleate cell. **Fig. 54**, bi-nucleate cell and tri-nucleate cell. **Fig. 55**, two metaphase plates in a bi-nucleate cell. 35, 36, 49, 50, 51, 53, 54, 55; $\times 600$. 52; $\times 900$. 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48; $\times 1200$. (*S. Makino photo.*)



Makino, S. & T. H. Yosida : Abnormal Mitosis in the Yoshida Sarcoma.



Makino, S. & T. H. Yosida : Abnormal Mitosis in the Yoshida Sarcoma.



Makino, S. & T. H. Yosida: Abnormal Mitosis in the Yoshida Sarcoma.

Cytological Studies on Cancer

I. Morphological and Statistical Observations on the Abnormal Mitosis in Tumor Cells of the Yoshida Sarcoma Through a Transplant Generation¹⁾

By

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(With 55 Figures in 8 Plates)

Introduction

Nowadays, the cytology of cancer, especially in the field of the chromosome study, from both morphological and physiological standpoints, has attracted particular concern of many investigators of tumor pathology concomitant with biological interest, primarily because the fundamental ground of cancer research may be built up with cytological phenomena. Heretofore, a considerable amount of work has been done in the field of cancer cytology; a high mitotic rate and a high frequency of abnormal division have been described as almost universal phenomena in tumors. The papers published by Goldschmidt & Fischer (1929), Lewis & Lockwood (1929), Kemp (1930), Ludford (1930), Levine (1931), Ishibasi et al (1931), Alexenko & Natansohn (1932), Amano & Ando (1940), Biesel et al (1942, 1945), Hauschka (1945-1948), and Casabona (1948) are important reports of cytological studies carried out with the tumors of rats and mice. Beginning early with the work of Hansemann published in 1891, Galeotti (1893), Heiberg & Kemp (1929), Levine (1930), Picon (1930), Winiwarter & Oguma (1930), Ohta (1933), Papanicolaou (1942, 1943), Barigozzi (1947), Barigozzi & Dellepiane (1947), Koller (1947), Barigozzi & Grattarola (1950), Polli (1950), Timonen (1950), Timonen & Therman (1950) and Therman & Timonen (1950) have made excellent contributions to the cytology of human cancer. A review of older literature has been given by Politzer (1935).

1) Contribution No. 254 from the Zoological Institute, Faculty of Science, Hokkaido University, Sapporo, Japan.

In reviewing the previous studies, it is evident that the attention of most of these authors was attracted to the variation of the chromosome number, various mitotic abnormalities and some related phenomena occurring in tumor cells. It is important and desirable to make inquiry into the behavior of tumor cells or the karyological changes occurring in them during the course of the growth of the tumor.

Unfortunately, for the purpose of cytological study the stroma-forming solid tumors, such as those removed from human bodies or those naturally or experimentally developed in rats and mice, long used as material by previous workers, have proved unfavourable. In addition, many technical difficulties associated with the classical paraffin method prevent the progress of study in this field to a considerable extent. Thus our knowledge on the cytology of cancer is much less satisfactory than is that in any other fields of cancer research. While referring to reports recently published by T. Yshida and his co-workers (1944, 1949) concerning the so-called Yoshida sarcoma, the authors received an impression that the latter sarcoma provides a number of favorable conditions for the cytological investigation. The Yoshida sarcoma, an ascites tumor, grows in the peritoneal cavity of white rats in a form of fluid tumor and is capable of successive transplantations. It allows of microscopical tracing of the behaviour and condition of tumor cells through every developmental stage of the tumor in a transplant generation, with a simple smear technique. A series of studies to be published hereafter will be carried out mainly on this sarcoma. Previously, some works on cytology of this tumor have been published, and fragmental evidences have been reported (Yosida & Makino 1949, Yosida 1948, 1949, a, b, Atsumi 1949, Sato 1950, Hirono 1951, Makino 1951).

The authors take much pleasure in expressing their sincere gratitude to Emeritus Professors, Dr. Kan Oguma and Dr. Yoshinari Kuwada for their important criticisms and helpful advice. Thanks are also due to Professor Tomizo Yoshida for advice and kind aid in connection with this work. Further they are also indebted to Miss Kyoko Kanō who kindly supplied some unpublished data along with some drawings and to Mr. Tatsuya Tanaka for friendly assistance. It is our pleasant duty to record here that this work is a part of studies having been facilitated by financial aid from the 'Kagakukenkyū Fund' and also the 'Kagakushikenkenkyū Fund' both from the Ministry of Education.

General characteristics of the Yoshida sarcoma

General accounts of the Yoshida sarcoma were rendered in detail by Yoshida (1944, 1949) who had established this tumor as a strain. But, it may be useful to make here some mention of the characteristic feature of this tumor. According to Yoshida (1949), the Yoshida sarcoma originally developed in an albino rat which had been fed with o-amidoazotoluene for 3 months and then applied cutaneously with potassium arsenite solution for about 4 months. The tumor ascites is of milky appearance and 1 cc of it contains approximately one million

tumor cells in a state of suspension. The transplantation can readily be made by injection of a small amount of tumor ascites into the peritoneal cavity of a new rat, with the aid of a sharply pointed glass pipette (Yoshida 1949). By this simple method, successive transmissions of the tumor have been made from rat to rat. After transplantation the tumor cells rapidly proliferate chiefly in the abdominal cavity of the new host, with a remarkable increase of ascites. The tumor-bearing animal dies in 12 days on the average.

Viewed from the cytological standpoint, the Yoshida sarcoma has many advantages for study as follows:

- 1) The tumor cells are surprisingly larger in size than any blood cells or other somatic cells occurring in the peritoneal cavity. This renders microscopical observation extremely easy.
- 2) The developmental condition of tumor cells in the growth of the tumor, and the process of their division can easily be observed in a single tumor animal at any time desired, or can be traced successively through the duration covering the time just after transplantation until the death of the tumor animal.
- 3) The simple and rapid smear method by the application of Giemsa's stain or acetocarmine allows close study for cytological purpose.

Technique

As mentioned above, the Yoshida sarcoma proved to be a favourable material for the cytological study in that the material for examination is available obtained at any time when wanted with a fine pipette without dissecting the animal, and further that the smear preparations are available for the chromosome investigation with satisfactory results. In the following-described investigations, both the Giemsa's azur-eosin-methylenblue solution for blood smear and the temporary smear technique with acetocarmine were employed. The procedure of Giemsa's method is thus; a small amount of ascites containing tumor cells is smeared on a slide from a droplet of the ascites which was obtained by inserting the glass pipette into the abdomen of the tumor animal, and fixed with absolute menthol for a few minutes. The slide is then allowed to dry and stained with Giemsa's solution for 40-60 minutes. After staining the preparation is washed with distilled water until properly differentiated and then blotted dry. In the acetocarmine smear, the fresh ascites smeared on a slide is directly covered with acetocarmine. It is desirable to leave the smear for about 20 minutes before applying the cover-slip. To seal the cover-slip, a new sealing medium is to be used (Makino & Nishimura 1952). The substitution either of acetic orcein as recommended by La Cour (1941) or of acetic gentianviolet as advised by Tanaka (1951) for acetocarmine, is likewise available for study with satisfactory results respectively.

The Giemsa-preparations are useful for the diagnostic observation of tumor cells, since by this method the criteria for morphological and physiological changes of tumor cells are to be obtained. But, this method is disadvantageous for the research of chromosomes; the cells become flattened by drying up the smear material

and this causes the shrinkage of cells showing artificial cohesion and irregular deformation of chromosomes. However, the acetocarmine smear technique can be employed with several advantages in the chromosome study, of which the most important are its simplicity, speed of procedure, and the results in both preservation and staining of chromosomes. Both cells and chromosomes are strikingly free from shrinking. The chromosomes prepared with this technique are observed with a well-defined, sharp outline and extreme clearness of appearance. Acetocarmine can be successfully substituted for acetic orcein or acetic gentianviolet as noted above.

The albino rats (*Rattus norvegicus*) which are derivatives of the strain from the Wistar Institute and have been bred pure in our laboratory were exclusively employed as host animals for the transmission of the tumor.

Observations

1. Descriptions of mitotic abnormalities in tumor cells

The tumor cells are very prominent by reason of being larger in size than any other cells observable in the peritoneal cavity. The nuclei are characterized by oval or kidney shape, or sometimes bilobed appearance, and locate on one side of the cell (Fig. 35). Conspicuous bodies, generally two or more in number, are visible within the nuclear body. Probably they are nucleoli because they are basophilic and negative to Feulgen reaction. In the wider space of the cell, distinct azurophilic granules make their appearance in cytoplasm arranging in a remarkable rosette form, when stained with Giemsa's stain.

It has frequently been reported by many investigators of tumor cytology that a high mitotic rate and a high frequency of abnormal division are almost universal phenomena in tumors (*cf.* Politzer 1935). The same can be said as to the Yoshida sarcoma here concerned. The smears prepared at any time from the ascites of the tumor animal exhibit a number of dividing tumor cells as well as various types of abnormal mitosis. A considerable daily variation occurs in the rate of cell division after transplantation. During about the first day after the transplantation of the tumor, the majority of the tumor cells undergo degeneration in the peritoneal cavity of the new host. The dividing cells are few in number. Afterward the division rate of cells progressively increases with time, and by the 5th or 6th day after transplantation, that is, in the middle part of life span of the tumor animal, the division of tumor cells is found at the highest frequency. Towards the latter part of the life span, that is, ten or more days after transplantation, the frequency of dividing cells decreases, while the cells in the course of degeneration proportionally increase. Throughout the course of the growth of the tumor, abnormal mitoses were observable at a high frequency showing a great variation in details of type.

In view of the fact that mitotic abnormalities are almost of common occurrence in tumors generally, it seems necessary to describe and illustrate in detail

these mitotic abnormalities in order to analyse the causes underlying them. Of the mitotic abnormalities¹⁾ occurring in the Yoshida sarcoma, the following types are remarkable :

Stickiness of the chromosomes is common, especially observed in the cells which are in disintegration (Figs. 1, 36). The chromosomes exhibit stickiness in more or less degree and fuse at points of contact specially at the ends. It is undoubtedly these sticky chromosomes that form chromatic bridges at anaphase stretching between the poles (Fig. 2), and lagging behind the regularly separating chromosomes (Fig. 40). Externally the stickiness seems to be dependent on cohesion of matrical substance of the chromosomes. Sometimes it happens that there is a loss of chromosome material in one of the daughter cells, while excess of them – namely, a partial doubling of chromosomes – in the other, occurs as a result of incomplete separation caused by the stickiness of chromosomes. Also, the fragmentation of the chromosomes seems, in most cases, to take place in close connection with the bridge-formation of sticky chromosomes. The size of fragments shows a great variation. Also, some chromosomes may be caught between the bridges, in which case they will be excluded from the nucleus. Thus, irregular and incomplete separation of chromosomes results from stickiness, and this gives rise to cells with externally variable chromosome contents. Extreme stickiness completely prevents the separation of the telophase chromosomes and this makes the cell division incomplete. In some cases, the daughter nuclei, after full division, remain associated by connecting chromosomal bridges (Fig. 43). The coalescence or irregular agglutination of chromosomes, a feature common in degenerating cells, is probably attributable to the extreme case of stickiness (Fig. 37). The abnormal swelling of chromosomes is also one of the noticeable changes here observed. When the swelling advances, the deformation of chromosomes into unusually rounded bodies results (Figs. 3, 38). The breaking down of the spiral chromonemata within chromosomes is also commonly observed (Figs. 4, 5, 39). Obviously, such a cell undergoes degeneration at metaphase without advancing further. These abnormalities have been considered to be a result of the abnormal metabolic change within the chromosomes.

Atypical arrangement of chromosomes at metaphase is of rather common occurrence in tumor cells. The chromosomes may be irregularly scattered about on the equatorial plane instead of taking the regular radial arrangement, or clumped together occupying an eccentric area (Fig. 6). Often the metaphase displays some chromosomes lying extruded outside the nuclear plate (Figs. 8, 42). The lagging chromosomes found at anaphase seem mostly to result from such extruded elements. Sometimes the chromosomes lie more contracted than usual.

1) Preliminary notes on this point were published by Yosida (1948), Makino and Yosida (1949), and Makino (1951), but in the present report more detailed accounts recently obtained will be given.

The occurrence of hollow metaphase plates in which the centre is without chromosomes is likewise a remarkable abnormal feature observable in tumor cells (Fig. 7). There are some intermediate configurations between normal metaphase plates and completely hollow plates. Evidently, hollowness is governed neither by a small number of chromosomes nor by the sticky nature of the chromosomes. It is obvious that these abnormalities are largely correlated with the deficiency of the spindle mechanism.

Lagging of the chromosomes during anaphase is a phenomenon of relatively common occurrence in tumor cells (Figs. 9, 10, 41). The laggards being 1 or 2 in number are most usual, but there are cases which show 5, 6 or even 10 laggards. The laggards may include the chromosomes undergoing non-disjunction, as well as those lying off the metaphase plate. The size of laggards shows also a wide variation. The lagging elements are either included in the separating chromosomes or excluded outside the daughter nuclei. The latter case is very common; it follows either the elimination of laggards or the formation of the micro-nucleus. It is a rather common phenomenon that the multipolar divisions, at least in anaphase, contain lagging chromosomes. Obviously, lagging of chromosomes during anaphase results in nuclei in which some chromosomes are absent, or in which extra, additional chromosomes are included. Unequal distribution of chromosomes at anaphase, or a migration of chromosomes in different numbers to poles, is also a pronounced feature (Figs. 11, 12). Though this phenomenon is often observed in giant cells undergoing polypolar division, it by no means always concerns multipolarity or polyploidy. It can be abundantly seen in the bipolar mitosis. Unequal distribution of chromosomes during anaphase is probably due to the failure of synchronization in separation of chromosomes, and it is usually brought about in close association with partial breakdown of the spindle mechanism.

The formation of the restitution-nucleus is indicated by the occurrence of polyploid cells with well-balanced chromosomes which show the regular metaphase arrangement and the normal bipolar spindle. Rather frequently, the restitution-nucleus assumes a bilobed appearance with two lobes of similar or dissimilar outline and remains associated by a narrow connection (Figs. 13, 14, 43, 44).

The multipolar mitosis occurs in a rather large proportion of cases as a remarkable feature of this sarcoma (Figs. 15, 16, 17, 19, 45, 46, 47, 48). It is seen either in actively dividing subdiploid or hypoploid cells or in giant cells. Although the giant cells show usually several poles in division, multipolarity is by no means always restricted to polyploidy. One can observe very small cells which show tripolar spindle, or also polyploid metaphase plates of a normal bipolar nature. The most usual type of multipolar division is tripolar division (Figs. 15, 16, 22, 46); observations showed that 73 percent in the total of the multipolar mitosis were tripolar. Tripolar divisions are more frequent in subdiploid cells than in hyperploid cells. The tripolar division results in different numbers of chromosomes at

three poles. For example, Figure 15 shows a tripolar division which had taken place in a subdiploid cell; in one of poles are nearly $2n$ chromosomes, while the other two poles show about 25 and 11 elements, respectively (Fig. 15). Example of quadripolar mitosis given in Figure 17. Pentapolar as well as hexapolar mitoses are rather rare. In Figure 48 an octopolar anaphase is depicted; it is likewise rarely found. Multipolar divisions of the giant cell forming many spindles are not of uncommon occurrence; generally, they are too complicated to be analysed in detail (Fig. 18). Most of these multipolar divisions contain lagging chromosomes together with chromosome bridges stretching between poles. These divisions result in the irregular migration of chromosomes in different numbers to poles at anaphase. The multipolar division takes place not only in polyploid cells, but also in multinucleate cells. But, the latter seems to be of rather common occurrence. The multipolar metaphase plates generally show abnormal orientation of the metaphase chromosomes. Sometimes the chromosomes lie in two or more layers at metaphase (Figs. 19, 20, 21, 45, 47). These findings suggest that the multipolar spindles mostly originate from the multinucleate cells. Generally speaking, the multipolar divisions result in the production of either a multinucleate cell with variable numbers of chromosomes, or a many lobed restitution-nucleus. For example, in the tripolar division, there is a formation of not only three cells with different number of chromosomes (Figs. 15, 16), but also two cells one of which is a bi-nucleate cell and the other a mono-nucleate cell (Fig. 22). If the tripolar division takes place in the subdiploid cell, a cell with a reduced number of chromosomes would be produced. Figure 23 shows a tetrapolar division resulting in the formation of two cells, one of which receives three nuclei and the other a single nucleus. Figure 51 illustrates a very huge giant cell in which a many-lobed restitution-nucleus was formed. In the giant multipolar metaphases the chromosomes very often show the beginning signs of disintegration forming amorphous chromosome masses (Figs. 49, 50). It seems highly probable that when the cell has reached a certain size it becomes inviable.

Referring to the above data, it may be understood that for the change of the nuclear contents the multipolar divisions take significant part as direct or indirect causes, giving rise to the cells with the extremely variable chromosome numbers. Thus, the abnormalities of the cell progressively increase due to a change in the number, structure and behavior of chromosomes from cell to cell with the progress of division. In the next step, the normal cellular activity stops probably due to the deficient nuclei which have resulted from abnormal mitoses. Finally the cells become inviable. Generally the percentage of multipolar metaphases in the total is considerably higher than that of multipolar anaphases. For instance, out of 73 percent of the tripolar division, 64 percent was found to be in the metaphase stage. This seems to suggest that most multipolar metaphases never reach the anaphase stage. A comparable feature was reported in human cancers (Timonen

and Therman 1950).

A further pronounced and common feature is the occurrence of multinucleate cells. The bi-nucleate cell is most usual and very frequent in appearance. The two nuclei contained in such a cell are approximately similar in size and shape in many cases (Figs. 24, 53), with a few exceptions in which they are of dissimilar size (Fig. 54). Though not so abundant, tri-, quadri-, penta-, and hexa-nucleate cells were likewise met with (Figs. 25, 26, 52, 53). Occasionally, so-called giant cells were observed which are characterized by the presence of some ten or more nuclei containing a considerable amount of cytoplasm. It is a general feature that the nuclei contained in these multi-nucleate cells are strikingly different from one another in both size and shape, and sometimes even in stage. In many cases, the nuclei contained in a cell undergo fairly synchronous division each showing the same phase of division (Figs. 27, 55), but in a few cases the nuclei did not show agreement in the stages of division (Fig. 28). The bi-nucleate as well as tri-nucleate cells assumed such a configuration. Commonly, the multi-nucleate cells are closely associated with the development of a multipolar spindle. In the multinucleate cells the chromosome plates are usually formed with an abnormal orientation (Figs. 20, 21).

The daily frequency¹⁾ of the multinucleate cell was calculated through the life span (16 days) of a tumor-bearing rat on the basis of 2000 cells per day (32000 cells in total). The results are as follows; the bi-nucleate cells occur in 30.1%, the tri-nucleate cells in 1.3%, the quadri-nucleate cells in 0.3%, the penta- and hexa-nucleate cells in 0.1%, respectively. Through a transplant generation the frequency of the bi-nucleate cell was found relatively high in the early part of the life span, whereas multi-nucleate cells containing three or more nuclei tended to increase in number from the middle part to the latter part.

The numerical change of the chromosomes has been described by many previous investigators in various human cancers as well as in experimental tumors of rats and mice as an almost universal phenomenon in tumor cells (cf. Makino's list 1951). Various deviations of the chromosome number from the normal one (42) were encountered in the Yoshida sarcoma too, as a remarkable abnormality of common occurrence. The counting of the chromosome number made in a number of metaphase plates indicated that the chromosomes showed a wide numerical variation ranging from about 20 to more or less than 85. Hence, the lowest number so far obtained in this sarcoma approximates 20, while the highest one appears as about 85 (Figs. 29, 30), except in the giant cell. Between these two extremities, the numbers exhibit a rather gradational fluctuation. Here important and noticeable is the fact that cells carrying 40 to 41 chromosomes (Figs. 31, 32, 33), which are nearly approximate to the normal number of the host animal (*Rattus norvegicus*;

1) The data in this respect were furnished through the courtesy of Miss K. Kanô.

$2n=42$, Fig. 34), were most frequent in occurrence. These cells appear to show a definite mitotic rate through the whole period of the life span of the tumor animal. Around 40-41, the chromosome numbers fluctuate both upward and downward in a quite gradual way. No true polyploidy seems to occur, but the cells close to triploid or tetraploid numbers were rarely found (Fig. 30). The giant cells with a very high number of chromosomes such as about 200 or more make also occasional appearance. Exact counting of chromosomes was impossible in these cells, since the metaphase orientation of chromosomes is mostly abnormal showing commonly the multipolar spindle (Fig. 18).

As above mentioned, the abnormalities concern the behavior of the chromosomes, or the spindle, or both. Undoubtedly, most of them lead to degeneration and death of the cells. It needs to be emphasized that cells which are undergoing abnormal mitosis cannot continue active multiplication and therefore they contribute little to the growth of the tumor. Here, particular attention should be paid to the fact that the cells having the subdiploid chromosome number (40 or thereabouts) are always very frequent showing a definite mitotic rate (Figs. 31, 32, 33), and further that they are quite regular in the behaviour of chromosomes in the course of division. It is highly probable that cells containing the subdiploid chromosomes play a significant part in the growth of the tumor because of their regular mitotic activity. On this point, details will be reported in another paper of this series of studies.

By way of summary the mitotic abnormalities described above are recognized as remarkable cytological phenomena in the Yoshida sarcoma. Broadly considered, these mitotic abnormalities can be arranged into two main categories: namely *i*) abnormalities involving structural alterations in the chromosomes, and *ii*) those attributable to accelerated, suppressed, delayed or incomplete spindle formation. To the former category may belong: stickiness and coalescence of chromosomes, abnormal swelling or vacuolization of chromosomes, deformation of chromosomes into unusual irregular bodies, and disintegration of spiral chromonemata in chromosomes. Probably these abnormalities are attributed to a change or changes in the organization of chromosomes during mitosis, for instance to an excess of deficiently polymerized nucleic acid as emphasized by Koller (1947). Into the latter category fall these abnormalities: lagging of chromosomes at anaphase, non-disjunction of chromosomes, chromosome bridges at anaphase and telophase, irregular chromosome distribution at anaphase, hollow metaphase, scattering or displacement from normal arrangement of chromosomes at metaphase, some chromosomes lying off the equatorial plate, abnormal orientation of metaphase chromosomes, the formation of the restitution-nucleus and multinucleate cells, and multipolar mitoses. Obviously, almost all these abnormalities contribute as direct or indirect causes for the variation of chromosome numbers, resulting finally in highly complicated duplication of chromosomes as seen in the giant cells.

The abnormalities of these categories can almost all be brought about in normal cells by various therapeutic agencies or by experiments in plants and animals, for instance by subjecting cells to high or low temperatures, by treating them with chemicals or some hypotonic or hypertonic media, or by exposing them to X-rays or γ -rays. The similarities between the abnormalities which occur in cancer and those induced by these artificial means are very striking. This problem will be touched upon again elsewhere.

2. The frequency of mitotic abnormalities

In the foregoing section, a number of mitotic abnormalities occurring in the Yoshida sarcoma were described with respect to their general morphology. In

Table 1. Total frequency of mitotic abnormalities in tumor cells observed in a tumor rat through 9 days of its life.

Type of abnormalities	Frequency (%)
Division type	36.8
Aberrant type :	25.8
<i>Hypo- & hyperploid</i>	1.3
<i>Multinucleated & multipolar cells</i>	3.8
<i>Irregular distribution of chroms. at anaphase ; lagging and non-disjunction</i>	6.8
<i>Displacement and abnormal orientation of metaphase chroms. ; hollow metaphase</i>	4.2
<i>Deformation of chroms.</i>	0.4
<i>Slight stickiness of chroms. ; chromosome bridges</i>	2.9
Disintegration type	37.2
Total number of cells observ.	236
%	99.8

Table 2. Daily frequency of the mitotic abnormalities. A, from the same

Indiv.	Type	Life days	1	2	3	4	5	6
			%					
A	Division type	53.6	31.2	46.8	20.8	42.8	51.6	
	Aberrant type	26.8	28.1	19.1	33.3	32.1	25.8	
	Disintegration type	19.5	40.6	34.0	45.8	25.1	22.5	
B	Division type	15.5	32.5	29.2	33.4	—	23.4	
	Aberrant type	62.0	54.9	57.5	60.0	—	43.3	
	Disintegration type	22.5	12.6	13.3	6.6	—	33.3	
C	Division type	—	12.5	20.0	20.0	—	30.0	
	Aberrant type	—	56.2	67.0	30.0	—	56.7	
	Disintegration type	—	31.9	13.0	50.0	—	13.3	

this section are presented the results of observations carried out on the frequency of mitotic abnormalities in tumor cells studied through the whole life span of the tumor-bearing animal. A droplet of tumor ascites was obtained once a day from a certain tumor animal, and smears were made for microscopical examination according to Giemsa's technique. Based on these preparations daily observations were made on the frequency of abnormal mitosis in tumor cells. The data were obtained from the dividing cells alone.

For the sake of convenience in description the tumor cells were divided into the following three types :

i) *Division type* ; this includes the cells which seem to undergo regular division with active mitotic behavior, showing the subdiploid chromosome numbers, 40 or thereabouts.

ii) *Aberrant type* ; to this type belong the cells showing many abnormalities, as described in the foregoing section.

iii) *Disintegration type* : in this group are included the cells where disintegration processes are seen in progress. They cover abnormalities such as coalescence or clumping together of chromosomes, abnormal vacuolization or swelling of chromosomes, breaking down of spiral chromonemata within chromosomes and giant cells with amorphous masses of chromosomes.

Firstly, the total frequency of abnormal mitosis occurring in tumor cells was observed in a certain tumor rat through its whole life span (9 days in this case), based on the daily material. The numbers of cells thus obtained were calculated in percentage on the basis of the total number of observed cells. The results were arranged in the three types above mentioned, as given in Table 1.

Next, the daily frequency of the mitotic abnormalities in tumor cells was observed based on the same material as used in Table 1. The results were given in Table 2, together with two other examples obtained from another material. As indicated in Table 2, the daily frequencies of the mitotic abnormalities furnish

material as Table 1. B and C, based on the material from two different individuals.

7	8	9	10	11	12	13	14	15	16
0 20.6 79.3	0 25.0 75.0								
21.5 75.2 3.3	20.0 65.8 14.2	6.6 70.1 23.3	— — —	3.4 38.0 58.6	0 20.0 80.0	16.0 28.0 56.0			
13.3 66.7 20.0	10.7 55.6 33.3	11.1 55.6 33.3	16.6 53.4 30.0	17.2 65.6 17.2	6.9 55.5 37.6	3.7 59.3 37.0	13.3 56.7 30.0	7.1 50.0 42.9	3.3 51.5 45.0

an interesting and significant feature regarding the growth of the tumor. The frequency of cells belonging to the division type gradually increases from the early part of the life span of the tumor animal toward the middle part, but decreases passing to the latter part. Cells of the aberrant type, however, exhibit the highest frequency of all during through the middle part of the life span betraying a decreasing tendency toward the latter part; those of the disintegration type are exclusively high in frequency in the latter part along with their considerable occurrence in the early part.

Based on the above data, the cycle of tumor cells in the development of the tumor can be described as follows: the tumor ascites inoculated in the peritoneal cavity of the new host probably contain cells of three types. The cells of the division type multiply through active mitosis and increase gradually in number during the period from the early part of the life span to the middle part together with the gradual increase of ascites, while those of the aberrant type tend to decrease during the early days, due to degeneration through abnormal mitosis. At the same time, the increase of cells of the disintegration type is brought about during the corresponding period. At the middle part of the life span when the most active malignant growth of the tumor is attained, the mitotic cells in the ascites are mostly of the division type. Then, the cells of the division type decrease in number with the passage of time. This decrease of cells of the division type corresponds to the increase of aberrant cells as well as of disintegrating cells. Most probably, this is due to the transition of cells of the division type into those of the aberrant or disintegration types. Obviously, the latter are unable to continue division. Hence, therefore, in the latter part of the life span, the majority of dividing cells are abnormal, and the disintegrating cells appear at the highest frequency. In the latter part, however, most cells of the division type remain inactive persisting in the resting condition. In reality, among degenerating cells, there occur a large number of resting cells which are characterized by small amount of cytoplasm and basophilic nuclei. The evidence presented suggests that the proliferation of tumor cells in the host by successive transplantations may primarily be attributable to the multiplication of cells of the division type, contributing to the growth of the tumor. This is one of the most important items pertaining to the mechanism of the malignant growth of the tumor, and therefore demands further confirmation of the fact. It would be premature to attempt to reach final conclusions as to the phenomenon from the results of the present investigation.

Before finishing, it may not be out of place to state here the hydration and dehydration theory on the cause of abnormal mitosis postulated by Kuwada (1937) and his followers. According to Kuwada, a change of water relation in the cell plays an important role in the production of mitotic abnormalities. Based on this theory, some mention will be made on the mitotic abnormalities occurring in the tumor. It has been shown that most of the mitotic abnormalities of common occur-

rence in nature and in experiments are regarded as being connected largely with the disturbance in normal water relation in the cell (Kuwada 1937, Shinke 1939, Sigenaga 1949, Nakamura and Makino 1950, Kanô 1951). The similarities between the abnormalities occurring in cancer and those induced by treating the cells with hypotonic or hypertonic media are very striking (Shinke 1939, Sigenaga 1949, Kanô 1951). As described in the foregoing pages, in this sarcoma tumor cells of the division type continue an active multiplication through a regular course of mitosis during the time when the peritoneal fluid of the host is still in probably undiseased condition. But, by the time when the peritoneal condition becomes unfavourable in the middle part of the life span due probably to the remarkable accumulation of the tumor ascites, cells of both aberrant and disintegration types increase rapidly with time. The accumulation of the tumor ascites, which consists in the greater part of degeneration products of tumor cells, probably brings about the change in viscosity of ascites. This change in the viscosity of ascites, in which tumor cells occur in a form of suspension, would disturb the water relation in tumor cells, if not directly, then in more or less degree as response to the osmotic pressure in the surrounding medium. This may be followed by changes of normal spindle mechanism or by the structural alteration of chromosomes due to dehydration or hydration processes, thus leading to various mitotic abnormalities. In addition, the substance produced by degeneration of tumor cells would contain something which acts as a mitotic poison for tumor cells. The sudden increase of abnormal mitotic figures of tumor cells in the latter part of the life span is very suggestive of this probability. We will have an opportunity in the near future to touch again this problem on the basis of the data accumulated in various experiments.

Summary

The Yoshida sarcoma here used as material is an ascites-sarcoma which grows in the peritoneal cavity of white rats (*Rattus norvegicus*) in a form of malignant fluid tumor. Successive transmission is carried out with ease by intraperitoneal inoculation of a droplet of the ascites containing tumor cells; the tumor cells rapidly multiply in the new host with a remarkable production of ascites, leading to the death of the tumor-bearing animal in 12 days on an average. The Yoshida sarcoma possesses many advantages for cytological investigation.

Various types of mitotic abnormalities occurring in the Yoshida sarcoma and the frequency of their occurrence have been described in this paper. On the basis of the daily frequencies of mitotic abnormalities observed through a transplant generation, the view was expressed that tumor cells which are undergoing regular mitosis with well-balanced subdiploid chromosomes primarily contribute to the growth of the tumor.

From the fact that striking similarities exist between the abnormalities occurring in the tumor and those induced by treating the cells with various chemicals,

or by hypotonic or hypertonic media, a tentative statement was made that a change in normal water distribution in the cell may be regarded as a cause of the mitotic abnormalities occurring in the tumor.

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Explanation of plate VII

All are camera-lucida drawings of tumor cells, $\times 1300$.

Fig. 1, stickiness of chromosomes. **Fig. 2**, chromosome bridges at anaphase. **Fig. 3**, deformation of chromosomes into rounded bodies. **Figs. 4-5**, breaking down of spiral chromonemata within chromosomes. **Fig. 6**, irregularly scattering of chromosomes in the metaphase plate. **Fig. 7**, hollow metaphase. **Fig. 8** showing some chromosomes lying extruded outside the equatorial plate. **Figs. 9-10**, lagging chromosomes at anaphase. **Figs. 11-12**, unequal distribution of chromosomes at anaphase. **Figs. 13-14**, formation of restitution-nucleus. **Figs. 15-16**, tripolar mitoses. **Fig. 17**, quadripolar mitosis.

Explanation of plate VIII

All are camera-lucida drawings of tumor cells, $\times 1300$.

Fig. 18, multipolar divisions in a giant cell. **Figs. 19-20**, tripolar mitoses, metaphase. **Fig. 21**, quadripolar mitosis, metaphase. Two equatoroal plates lie at about right angle. **Fig. 22**, telophase of a tripolar mitosis, forming a binucleate cell. **Fig. 23**, telophase of a quadripolar mitosis forming a tri-nucleate cell. **Fig. 24**, bi-nucleate cell. **Fig. 25**, trinucleate cell. **Fig. 26**, tetra-nucleate cell. **Fig. 27**, two metaphase plates in a bi-nucleate cell. **Fig. 28**, showing asynchronous division in a bi-nucleate cell. **Fig. 29**, metaphase plate showing 22 chromosomes. **Fig. 30**, metaphase plate showing 80 chromosomes (subtetraploid). **Figs. 31-33**, metaphase plates showing well-balanced subdiploid chromosomes; 41, 42 and 41 elements in each. **Fig. 34**, metaphase plate of a blood cell showing 42 chromosomes.

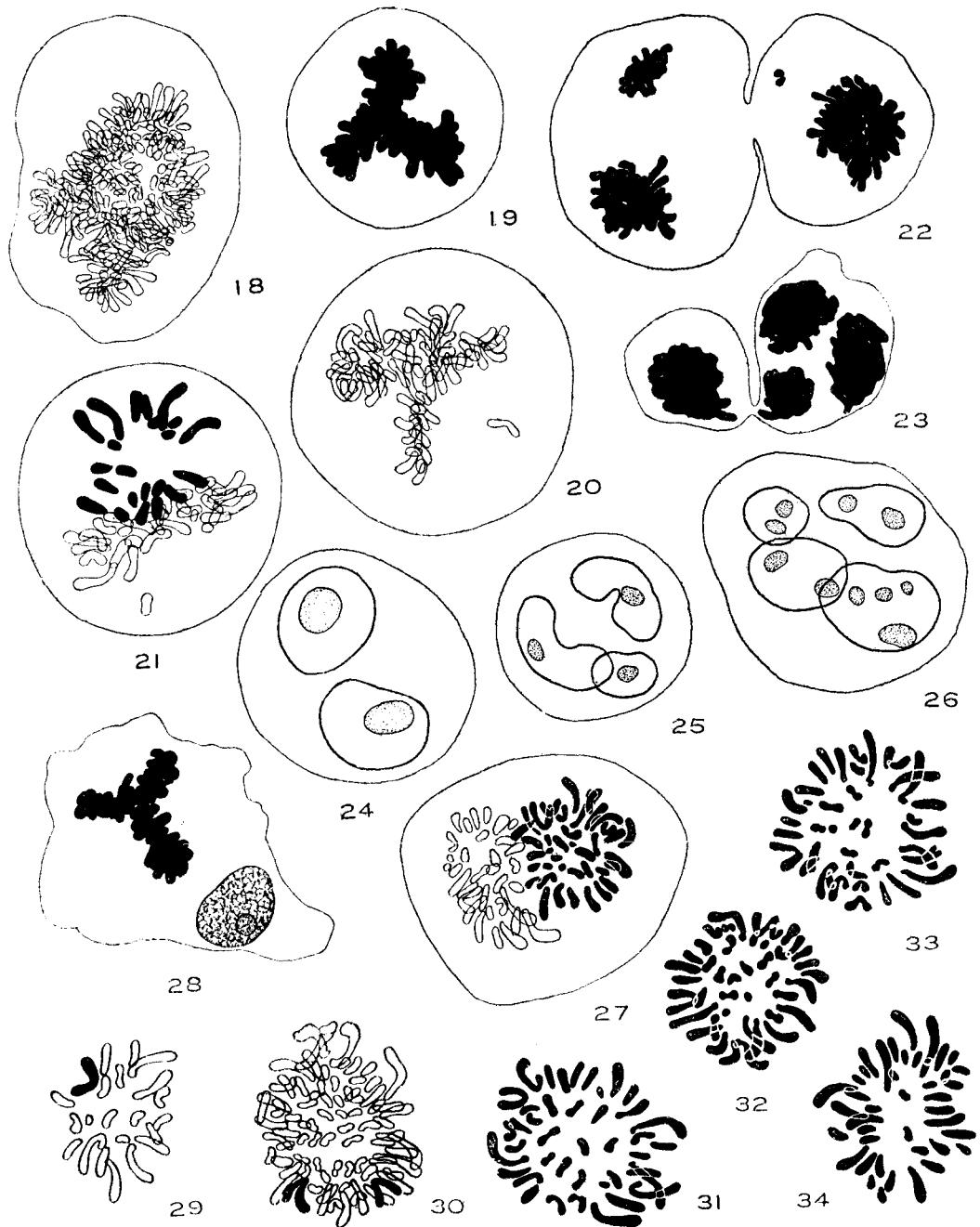
Explanation of plate IX

All are photomicrographs of tumor cells.

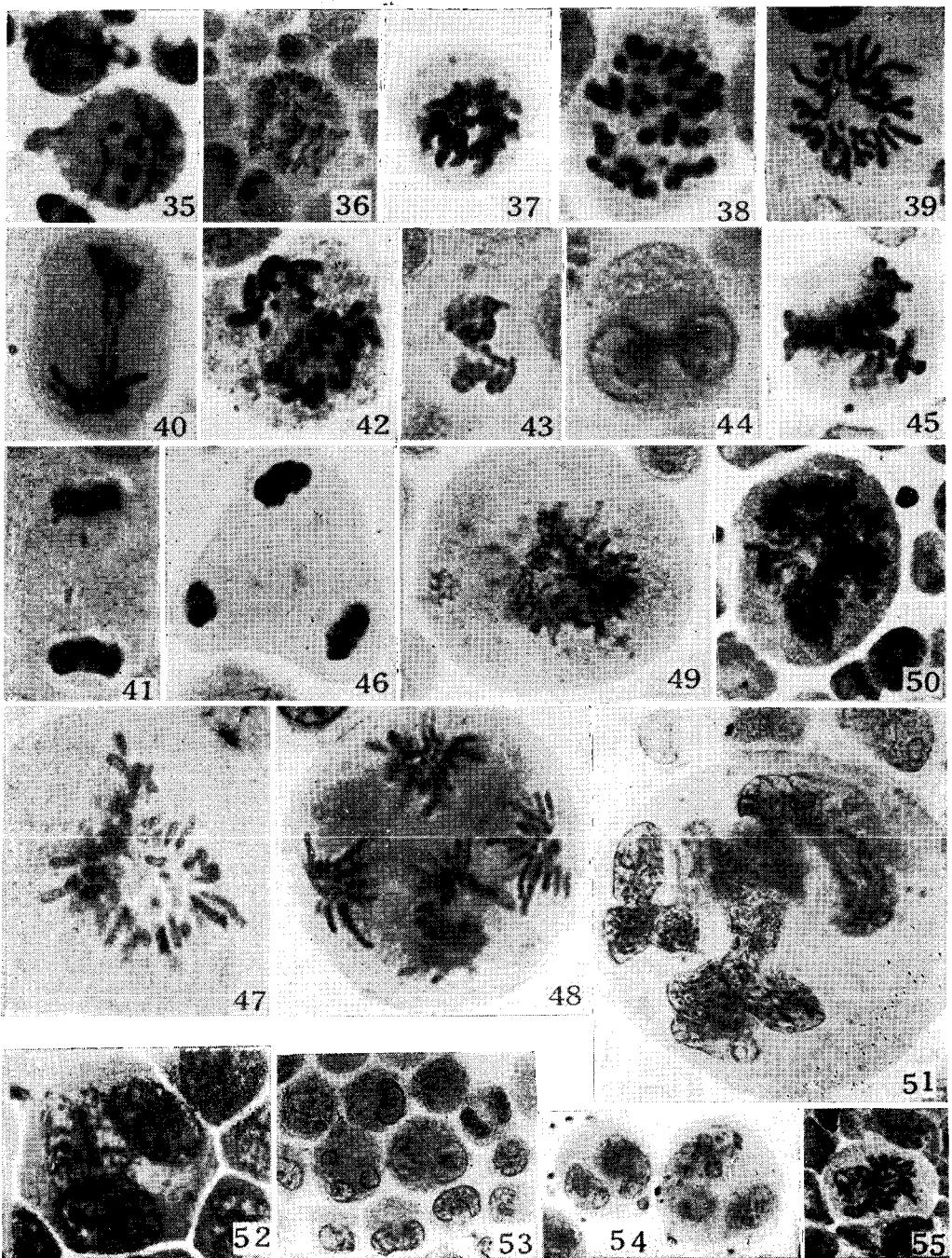
Fig. 35, tumor cells at resting stage, showing remarkable nucleoli in nuclei. **Fig. 36**, stickiness of chromosomes. **Fig. 37**, coalescence of chromosomes. **Fig. 38**, deformation of chromosomes into rounded form. **Fig. 39**, breaking down of spiral chromonemata. **Fig. 40**, chromosome bridges at anaphase. **Fig. 41**, lagging of chromosomes at anaphase. **Fig. 42**, showing some chromosomes extruded outside the equatorial plate. **Fig. 43**, formation of restitution-nucleus. **Fig. 44**, two nuclei with narrow connection. **Fig. 45**, tripolar metaphase. **Fig. 46**, tripolar telophase. **Fig. 47**, tetrapolar metaphase. **Fig. 48**, octopolar anaphase. **Figs. 49-50**, giant cells showing amorphous masses of chromosomes. **Fig. 51**, giant cell containing lobulated nuclei. **Fig. 52**, tri-nucleate cell. **Fig. 53**, showing bi-nucleate cells and tri-nucleate cell. **Fig. 54**, bi-nucleate cell and tri-nucleate cell. **Fig. 55**, two metaphase plates in a bi-nucleate cell. 35, 36, 49, 50, 51, 53, 54, 55; $\times 600$. 52; $\times 900$. 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48; $\times 1200$. (*S. Makino photo.*)



Makino, S. & T. H. Yosida : Abnormal Mitosis in the Yoshida Sarcoma.



Makino, S. & T. H. Yosida : *Abnormal Mitosis in the Yoshida Sarcoma.*



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