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Title	Cytological Studies on Cancer : II. Daily Observations on the Mitotic Frequency and the Variation of the Chromosome Number in Tumor Cells of the Yoshida Sarcoma Through a Transplant Generation (With 37 Figures in 2 Plates)
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Citation	北海道大學理學部紀要, 10(3-4), 225-242
Issue Date	1951-12
Doc URL	https://hdl.handle.net/2115/27094
Type	departmental bulletin paper
File Information	10(3_4)_P225-242.pdf

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Cytological Studies on Cancer

II. Daily Observations on the Mitotic Frequency and the Variation of the Chromosome Number in Tumor Cells of the Yoshida Sarcoma Through a Transplant Generation[1)]

By

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(With 37 Figures in 2 Plates)

The mechanism of the malignant growth of the tumor is a matter of fundamental importance as well as of primary concern in cancer research. Although surprising advance has recently been made in this field of cancer pathology as presented in a good many important reports, the state of knowledge still seems inadequate for clarifying many important problems growing up around this subject. A new approach to these fundamental problems, however, will be opened to a considerable extent by research in the field of cytology, especially by the study of chromosomes of the tumor ; much confusion underlaying these points has remained unsolved.

Based on the daily analysis of the frequency of mitotic abnormalities occurring in the Yoshida sarcoma, the findings reported in the previous study suggest that cells which are undergoing a regular division with well-balanced subdiploid chromosomes play a significant role in the growth of the tumor (Makino and Yosida 1951). The data presented in that paper were obviously scanty ; therefore, it was premature to attempt any conclusive statement concerning the phenomenon. The present study was undertaken with a view to further confirm the previously reported evidence, through close observations of the mitotic rate of tumor cells. Observations were carried out in close connection with investigations of the chromosome constitution in the Yoshida sarcoma.

The hereunder described observations were made using smear preparations

1) Contribution No. 255 from the Zoological Institute, Faculty of Science, Hokkaido University, Sapporo, Japan.

daily prepared according to the procedure as follows : The smear was made from a droplet of the tumor ascites once a day from a certain tumor rat through its whole life, starting with the first day after transplantation up to the last day at intervals of about 24 hours. The slides thus prepared were stained after both Giemsa's and acetocarmine methods. Accurate observations concerning chromosomes were made exclusively in the acetocarmine preparations. The present study was based on material derived from tumors transplanted in three different individuals of white rats belonging to the purely bred Wistar strain.

The authors' cordial thanks are due to Mr. Tosihide H. Yosida and Mr. Tatsuya Tanaka for their kind assistance in the course of this study. This paper constitutes a part of studies carried out with the aid of grants from the 'Kagaku-kenkyu Fund' and also the 'Kagakushiken-kenkyu Fund', both out of the Ministry of Education.

General course of the growth of the Yoshida sarcoma in a transplant generation

By intraperitoneal introduction the ascites sarcoma grows in the new host and leads the diseased animal to death in 12 days on an average. General course of the growth of the tumor is as follows :

The tumor ascites inoculated in the new host in successive transplantations contains two types of tumor cells (Fig. 25). The one comprises relatively large cells generally characterized by a considerable amount of cytoplasm and a large nucleus of bilobed or kidney shape, or sometimes of very complicated form. Giant cells containing huge nuclei of bizarre outline are of frequent occurrence in this type. The other comprises small cells which are remarkable for their very small amonut of cytoplasm and a rather spherical, well-defined nucleus of compact appearance (Fig. 25) ; both the cytoplasm and nucleus are intensely basophilic in staining.

Following the transplantation of the tumor ascites, a great many tumor cells introduced into the peritoneal cavity of the new host seem to undergo degeneration, since the cells in the course of disintegration and those showing various abnormalities appear at a high rate (Fig. 24). The dividing cells are very few. The majority of these degenerating cells are large in size. The mitotic figures of tumor cells make their gradual appearance about 24 hours after transplantation. The number of dividing cells progressively increases with the passage of time. On the 3rd or 4th day after transplantation, the peritoneal fluid of the host seems to be in the most favorable state for proliferation of tumor cells and the latter proliferate very rapidly under this circumstance. Towards the middle part of the life span of the tumor animal, that is, on the 5th or 6th day the cells undergoing mitosis are seen to be most numerous. Then, they begin gradually to decrease with time towards the latter part of the life span. In correlation to the decrease of the

dividing cells, cells showing mitotic abnormalities and disintegration increase in frequency. The tumor ascites proportionally shows a remarkable accumulation. Nearing the last day of the host animal's life, dividing cells suddenly descrease in number. In striking contrast, the cells showing abnormalities as well as those of disintegration appear at a remarkably high rate. By this time the accumulation of the tumor ascites reaches an enormous amount with a great expansion of the abdomen of the tumor animal. Mingled with these degenerating cells, there occur in the tumor ascites a number of resting cells, which are characterized by their small size containing a small amount of cytoplasm and well-defined, basophilic nuclei (Fig. 26). The malignant growth of the tumor having reached this stage leads to the death of the host animal.

Observations

1. Rate of mitosis in a transplant generation

The rate of mitosis in tumor cells was observed with the daily material through the whole life span of certain tumor rats. For convenience of description, the entire life span of the tumor-bearing animal was divided into three parts, namely the early, middle and latter parts. Viewing the whole material the mitotic frequency in tumor cells is seen to show a slight variation day by day, and also according to individuals. But, there is observable a characteristic tendency in tumor cells of this sarcoma with respect to the mitotic rate.

The specimen here to be considered died on the 16th day after transplantation of the tumor. Two thousand tumor cells were observed every day through the entire period of the life of the tumor-bearing animal, and the daily frequencly of dividing cells covering those of late prophase, metaphase, anaphase and telophase, was calculated in percentage on the basis of the above data. The results are given in Table 1, and graphed in Chart 1.

Referring to the data presented in both Table 1 and Chart 1, it is apparent that the number of dividing cells shows a gradual increase from the early part continuing towards the middle part of the life span. The rate of mitosis forms a peak curve through the middle part, decreasing gradually towards the latter part.

Table 1. Daily frequency of mitotic cells in a tumor rat. 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	13	14	15	16
% of dividing cells	1.7	4.4	4.6	3.8	4.0	4.4	3.9	3.2	3.0	3.3	3.2	1.9	0.5	0.4	0.8	0.2

Another example likewise represents a similar result, described in the paper of Kanô (1951).

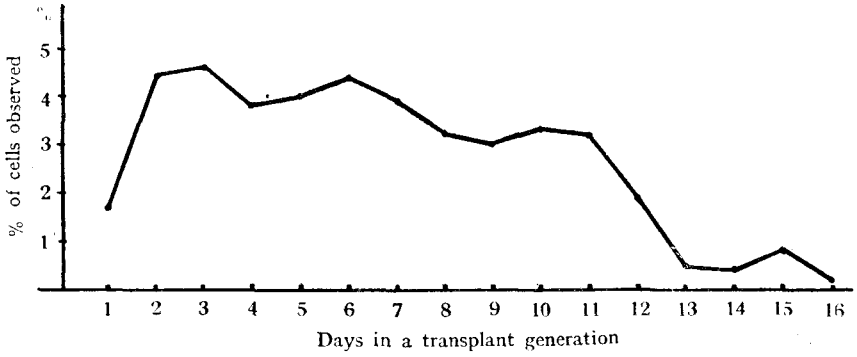

Chart 1. Graphical representation of the daily frequency of mitotic cells in a transplant generation of the tumor rat, based on the data in Table 1.

In the next observations based on the same material as above, attention was paid to the daily frequency of the cells with well-balanced subdiploid chromosomes having an ordinary metaphase configuration. The daily frequencies of the regularly dividing cells at metaphase, together with those of abnormal metaphase cells, were calculated on the basis of the total number of metaphase cells in the

Table 2. Daily frequency of the cells at metaphase, based on the same material as given in Table 1. The percentage was calculated on the basis of the total number of metaphase cells observed every day.

Days after transplantation	Regular cells at metaphase		Irregular cells at metaphase		Disintegrating cells at mataphase		Total number of metaphase cells observed	
	Number	%	Number	%	Number	%	Number	%
1	18	9.0	60	30.0	122	61.0	200	100.0
2	92	38 3	124	51.7	24	10.0	240	100.0
3	86	35.8	125	52.1	29	12.1	240	100.0
4	79	35.6	111	50.0	32	14.4	222	100 0
5	80	35.6	100	44.4	45	20.0	225	100.0
6	104	40.5	106	41.2	47	18.3	257	100.0
7	57	30.8	75	40.6	53	28.6	185	100.0
8	40	29.0	42	3.04	56	40.6	138	100.0
9	65	31.7	104	50.7	36	17.6	205	100.0
10	90	32 3	127	45.7	61	22.0	278	100.0
11	51	23.6	64	30.2	98	46.2	212	100.0
12 ≀ 16	15	8.5	71	42.5	81	45.5	167	100.0

daily observation. The results are given in Table 2.[1]

The data presented in Table 2 indicate that, on the first day after transplantation of the tumor the number of cells with well-balanced subdiploid chromosomes is extremely small, whereas cells showing abnormalities appear in a considerably large number. The frequency of cells with well-balanced chromosomes shows a remarkable increase with time, and during the middle part of the life span, namely on the 5th to 7th day after transplantation, they are found in an enormous number. It is by this time that the most active growth of the tumor is attained, with a pronounced increase of the tumor ascites. Towards the latter part of the animal's life, the chromosomally well-balanced cells show a gradual decrease in number, being replaced by cells showing mitotic abnormalities which show a gradual increase in number. On the 11th day, or later, after transplantation, the cells showing

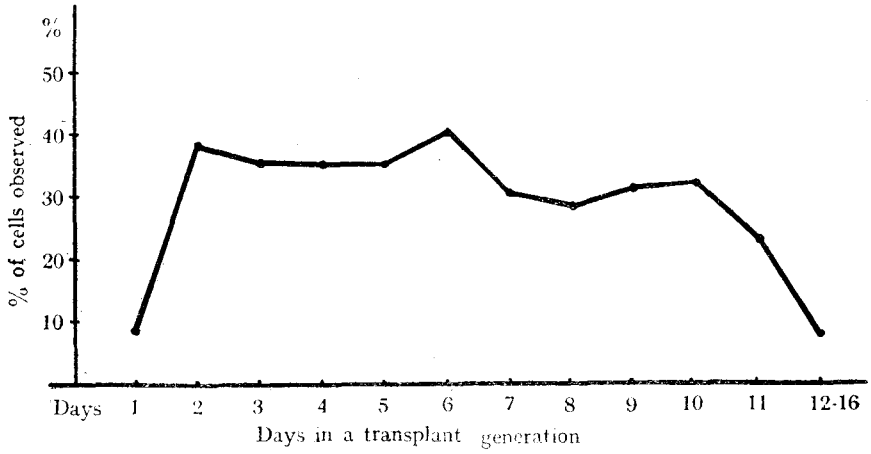

Chart 2. Graphical representation of the daily frequency of regularly dividing cells at metaphase, observed through a transplant generation, based on the data in Table 2. From the same material as used in Chart 1.

stickiness or coalescence of chromosomes, or disorganization of chromosomes appear at a strikingly high rate, in remarkable contrast to the low frequency of cells with subdiploid chromosomes.

1) In Table 2, the metaphase cells were classified into three types. Regular metaphase cells include the cells with a well-balanced subdiploid chromosomes, showing a regular feature and equatorial arrangement. The cells showing abnormal arrangement and aberrant number of chromosomes are designated as irregular metaphase cells. The disintegrating cells are those showing stickiness and coalescence of chromosomes, or disorganization of chromosomes at metaphase.

Table 1 indicated that the number of dividing cells shows a gradual increase from the early part continuing towards the middle part of the life span of the tumor animal, and decreases gradually towards the latter part. The data presented in Table 2 run parallel to the above ; that is to say, the frequency of chromosomally well-balanced subdiploid cells shows again a gradual increase from the early part, being high during the middle part, and decreases gradually towards the latter part of the life span. In other words, along with the increase of dividing cells, the frequency of cells with subdiploid chromosomes also increases, and the decreases of the former again responds to the decrease of the latter cells. This is well understood by reference to Charts 1 and 2, in which are found two curves running nearly parallel. Obviously, the cells showing mitotic abnormalities are unable to continue active multiplication and therefore they cannot affect the growth of the tumor. Thus, from the comparison of the data presented in Table 1 (and Chart 1) and Table 2 (and Chart 2), derived from the same material, the conclusion may be drawn that, among the dividing tumor cells, those with well-balanced subdiploid chromosomes play a significant role in the multiplication of cells in the growth of the tumor. To state the point otherwise, the multiplication of tumor cells in the new host in successive transplant generations is primarily attributable to the chromosomally well-balanced subdiploid cells. Obviously, these well-balanced cells show nothing unusual in either equatorial arrangement or constitution of chromosomes, and are quite regular in behavior just as occurs in normal cells.[1] Thus, the chromosomally well-balanced cells which are apparantly regular in respect to their chromosomes and in their behavior during mitosis contribute primarily to the growth of the tumor. Eventually, the results of the present observations agree with those of the previous study concerning the daily frequencies of mitotic abnormalities in this sarcoma.

2. Accounts on the chromosome number

In order to learn the chromosome number of the cells with well-balanced chromosomes having an ordinary metaphase configuration, which primarily are concerned with the growth of the tumor as above mentioned, the counting of the chromosomes was undertaken using the same preparations as employed in the above studies. Taking into consideration only the chromosomally well-balanced subdiploid cells with ordinary constitution and equatorial arrangement of chromosomes, the number of chromosomes was observed in the Giemsa-preparations, prepared every day through the whole life span of a tumor rat. The Giemsa-preparations, however, yielded no difinite results as for the chromosome number, so that only an approximate estimation of the number, such as 30-40, was possible in this case.

1) In this respect, see the next paper (Makino 1952).

The results of observations are shown in Table 3. It is evident from this table, that in the cells with a well-balanced metaphase configuration the chromosome number shows a small range of variation, and that cells with the chromosome numbers ranging from 30 to 40 are the most frequent of all. The data here obtained were confirmed by those coming from another specimen which likewise showed the same result, as given in Table 4. (In this case the chromosome counting was made

Table 3. Daily observations of the chromosome numbers in tumor cells through a transplant generation, from the same specimen as given in Tables 1 and 2. (Giemsa-preparations).

Days after transplantation	Chrom. Numbers				Total
	20—33	31—40	41—50	51—60	
	Number of cells observed				
2	12	18	1		31
3	5	21	4		30
4	7	18	4		29
5	13	29	1		43
6	7	23	6	1	37
7	6	15	2	1	24
8	10	13	2		25
9	9	19	10	2	40
10	9	21	8	2	40
11	10	22	3		35
12					
16	5	10			15
Total	93	209	41	6	349
%	26.6	60.0	11.7	1.7	100.0

Table 4. Chromosome numbers observed in a sample taken on the 5th day after transplantation of the tumor.

	Chromosome numbers			Total
	20—30	31—40	41—45	
Number of cells observed	7	63	10	80
%	8.75	78.75	12.5	100.0

in a sample taken on the 5th day after transplantation of the tumor in which dividing cells showed the highest frequency). Study of Table 4 indicates that the cells having 30-40 chromosomes accounted for 78.75% in a total of 80 metaphase cells under observation. Based on the above facts, it may be concluded that the majority of the chromosomally well-balanced cells contain 30-40 chromosomes,

so far as the rough counting based on Giemsa-preparations is concerned. Therefore, it can be said that probably the cells which primarily contribute to the growth of the tumor have the chromosome numbers ranging from 30 to 40.

Now, the necessity arises in the next step to get as accurate as possible counts of the chromosome number in tumor cells. However, the Giemsa-preparations are not useful at all for the exact study of chromosomes, because they yield some artifacts such as temporary fusion or abnormal swelling of chromosomes which all hamper close observation to a great extent. The acetocarmine smear method, on the contrary, proved to be excellent for the study of chromosomes, since by this method the chromosomes at metaphase are visible with a well-defined sharp outline showing no sign of irregular agglutination. The following observations were carried out with acetocarmine smear preparations exclusively. They were prepared every day with the tumor ascites secured from a new specimen different from that used in the above observations. This rat died on the 12th day after transplantation of the tumor. In observation, only clear metaphase cells were selected in which the adequate counting of the chromosome number is possible to a considerable extent. The daily variations of the chromosome numbers were observed through 12 days, from the first day after transplantation of the tumor up to the last day of life. The giant cells were disregarded. Even in good plates, an uncertainty of ± 1 could not be avoided in counting, except in very excellent ones. The results of this survey are arranged in Table 5. Referring to this table it is apparent that the number of chromosomes of tumor cells varies with a rather wide range : between about 22 for the smallest extremity and 80 for the largest one (Figs. 1-8). The variation of the number is rather gradational around a figure close to 40. On the whole, however, cells having chromosomes numbering less than 42 are more frequent than those numbering more. The latter cells are rather

Table 5. Daily observations on the chromosome numbers in tumor cells through a

Chrom. No. / Days	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
1				1					3			4	1	5	1		2		2	2	4	1		2				
2					1	2	2	2	3		5	6	7	7	9	7	6	5	8	6	5	1	2	1	1				
3	1	1							2	2	2	3	4	4	3	5	5	6	3	4	3	1							
4					1				2	4	5	4	3	2	6	7	5	5	7	5	1				1				
5							1		1		2	1	1	4	4	6	3	6	8	6	3	1	2						
6							1	1	2	1	2	2	2	2	5	4	4	6	2	5	3	1	1		1				
7						1						2		4	6	5	8	5	8	3	4	1		1					
8									1		1	1	1	1	1	3	8	6	9	11	5	1							
9										1			2	3	5	3	4	11	8	8	3								
10											2	2	1	3	5	5	6	8	7	4	3								1
11											1	1			2	3	4	6	12	11	4	2	2				1		
12											1	1	3	3	4	5	5	4	5	7	1								1
Total	1	0	1	1	2	3	4	3	14	8	21	27	25	38	51	53	60	68	79	72	39	9	7	4	3	0	1	0	2

infrequent. Also, true polyploidy seems to be very rare in occurrence or absent. But there appear very rarely subtriploidy showing ±60 (Fig. 7) and subtetraploidy having ±80 (Fig. 8). Special attention should be paid to the fact that the tumor cells showing chromosome numbers which range from ±35 to ±42 are very frequent for each day. Particularly, the cells with 38, 39, 40 and 41 chromosomes appear each day at a strikingly high rate (Figs. 2-6). Among them, cells having 40 chromosomes are the most frequent, and those with 41 chromosomes rank second. This fact is important in that most frequently the tumor cells contain the chromosomes very nearly approximate in number to those of the host, *Rattus norvegicus*, in which 42 diploid chromosomes were found in both germ and somatic cells (Makino 1942, 1943, Tanaka 1951). It is also remarkable that cells with a lower chromosome number than ±40 are more frequent than those with larger number than ±40.

In order to learn the daily frequency of the cells with the chromosome numbers 38-41, the necessary data were picked out from Table 5 and arranged in Table 6. In this table, the total sum of cells with 38-41 chromosomes is given for each day, and the percentage is calculated on the basis of the total number of cells observed. Since the data presented in Talbe 6 are based on the clear metaphase cells adequate for chromosome counting alone, it is not permissible to use them as a criterion for comparison with those of Table 3. The high percentage of the cells with 38-41 chromosomes during the latter part of the life span of the tumor rat as given in Table 6 is undoubtedly attributable to the absence of data concerning giant cells or cells undergoing disintegration. By comparison of Table 3 with Table 4, it is allowable to say that the daily occurrence of cells with 30-40 chromosomes is nearly in a parallel relation to that of cells with 38-41 chromosomes. This implies that the cells with the chromosome numbers between 30 and 40 (Table 3) may be largely replaced, if not entirely, with those which contain approximately 40 chromo-

transplant generation of the Yoshida sarcoma, based on acetocarmine preparations.

51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	Total
1				1																										30
	1					1										1			1											90
		1																												50
					1				1																					60
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0	1	1	2	1	2	1	0	0	2	1	0	0	1	0	1	1	1	0	2	0	1	2	2	1	1	0	0	0	1	620

somes. From this conception, and by reference again to the data of Tables 1 and 2, it follows that the tumor cells which are characterized by a well-defined, regular metaphase configuration, and which play a significant part in the growth of the tumor, have the chromosome numbers of approximately 40.

Table 6. Picked-out data from Table 5, showing a daily frequency of the cells with 38—41 chromosomes.

Days after transplantation	No. of cells with 38—41 chroms. and their percentage		Total of cells observed
1	6	20.0%	30
2	25	27.8	90
3	18	36.0	50
4	22	36.6	60
5	23	46.0	50
6	17	34.0	50
7	24	48.0	50
8	34	68.0	50
9	31	62.0	50
10	25	50.0	50
11	33	66.0	50
12	21	52.0	40
Total	279	45.0	620

Summing up, it may be said that, on account of their high frequency and a definite rate of division, the tumor cells with 40 chromosomes or thereabouts, primarily contribute to the growth of the tumor through their active proliferation. And further, the variation of the chromosome number takes place arround the number 40, showing a wide fluctuation from about 20 to approximately 80. As a whole the cells containing chromosome numbers lower than ±40 are more remarkably frequent than those with numbers larger than ±40.

Now, our particular interests concern the chromosome constitution of the cells which contain a well-balanced subdiploid set of chromosomes, 40 or thereabouts in number, and are responsible for the growth of the tumor. Observations by the senior author (S. M.) revealed that these cells carry a quite characteristic karyotype which is of this sarcome proper and again strikingly differentiated from that of the host, *Ratlus norvegicus* (Fig. 9), being characterized by a certain number of V-shaped elements (Figs. 2-6). Details will be given in the next paper of these serial studies.

Remarks

1. On the mitotic capacity in cells with reduced or increased chromosome numbers

It was pointed out in the foregoing observations that there is a group or

strain of cells which contain the chromosome numbers of 40 or thereabouts, and further that they are primarily responsible for the growth of the tumor on account of their strikingly high frequency and definite mitotic rate. It is of interest that the specific number ±40 here concerned is closely approximate to the normal chromosome number of the host in which the diploid number of 42 has been well established. Winge (1930) reported in the tar-carcinoma of mice that the cells with 36-40 chromosomes were most frequent (the normal number for mice being 40).

As already mentioned, the variation of the chromosome number in this sarcoma takes place around the specific number ±40. But, noticeably the cells with lower numbers than ±40 show larger frequency than those with higher numbers than ±40 (cf. Table 5). Particularly the cells having 36, 37, 38, 39, 40 and 41 chromosomes show a strongly increased frequency. This evidence is an indication that the cells with such chromosome numbers have a mitotic capacity exceeding that of other cells. Until recently, it has been considered that a nucleus with the complete chromosome number is necessary of the regular functioning of cells (Koller 1943, 1947), and that cells with deficient nuclei can survive only under very specific conditions (Barber 1941, Sax 1942), the life of such cells always being short (Koller & Smithers 1946). The Yoshida sarcoma here under consideration furnishes, however, an apposite example, since cells with reduced or increased chromosome number are still able to divide and to continue dividing. Similar evidence was presented by Koller (1947) in the human adenocarcinoma. These facts seem to imply that, because the cells remain active in spite of their deficient nuclei, the complete chromosome set is not necessarily required for the accomplishment of normal cell behavior. Koller (1947) interpreted the phenomenon by considering that mitotic activity is under cytoplasmic and not nuclear control. However, this assumption seems not adequate for the present case. The present authors are of opinion that the inner balance held between the nucleus and cytoplasm is necessary for the regular functioning of cells, namely that under the balanced condition the nucleus can control the cytoplasm to accomplish normal cell function, due probably to the co-operation between the former and the latter. So long as the inner balance between the nucleus and cytoplasm remains favourable, the cells have mitotic capacity. It appears likely that the normal nucleic acid cycle of the chromosomes can proceed, or remain active, within this limit. If the nucleus contain a certain set of chromosomes necessary for controlling the cytoplasm, or else for co-operation with the latter, it should permit mitotic activity without difficulty, even though the chromosome set is not always entirely complete. The case of the Yoshida sarcoma is an example strongly favouring this assumption. Because of their highest mitotic rate, as seen in Table 5, the cells with 40 chromosomes might be in the most well-balanced condition in the nucleus-cytoplasmic relationship. The frequency of the cells with chromosome numbers lower than 40, and that of the cells with higher numbers shows a gradual decrease, as the chromosome

number becomes either greater or less than 40. Probably, the inner balance of cells might increasingly be disturbed and therefore mitotic activity might progressively be interrupted, as the number varies above or below 40.

Koller (1947) further claimed that tumor cells may be dependent on each other in many respects, and that in undergoing mitosis, a specific substance necessary for chromosome synthesis is transferred from one cell to adjacent cells. For this reason, according to him, cells with a reduced chromosome number are able to divide. Also, there is another but similar view that nuclei with normal chromosome sets are capable of controlling the mitosis of other adjacent nuclei which lack a normal chromosome set (Svärdson 1945). These are interseting ideas for the interpretation of the phenomenon respectively, but they cannot be applicable for the present case, because the Yoshida sarcoma is a fluid tumor in which tumor cells always occur independently from one another in a form of a suspension without forming massive tissue.

Here is also another view involving a hypothesis concerning mitotic genes; namely, there are in the nucleus a series of genes which are required for the accomplishment of normal mitosis (cf. Svärdson 1945). The mitotic genes may either be concentrated to a few chromosomes or else each chromosome may have a set of mitotic genes. This assumption cannot be easily repudiated, since it must be of considerable importance in consideration of mitotic activity of cells with a reduced chromosome number. However, knowledge of these mitotic genes is exceedingly deficient. As yet very little is known about the various fields of activity of these genes (Mather 1942). Though nothing can be said at present, this hypothesis is also of considerable interest in connection with the theory of gene or chromosome mutation as connected with the origin of cancer.

As is obvious by reference to Table 5, the occurrence of a true polyploidy seems to be very rare or absent in tumor cells. But, the cells with subtriploid chromosome numbers such as ± 55 and ± 60, and those with subtetraploid numbers such as ± 75 and ± 80, are rarely found (Figs. 7-8). In the majority of polyploid cells, abnormal orientation of metaphase chromosomes and the formation of multipolar or incomplete spindle occurred very often (cf. the column of irregular metaphase cells in Table 2), so that the formation of a regular metaphase plate is interrupted. Of course, these obstruct a regular proceeding of cell division and lead to death of the cells, due to the numerous cytological abnormalities induced. Only the cells with a well-balanced chromosome number must form a regular metaphse plate, showing a normal orientation and arrangement of chromosomes. On account of the fact that the frequency of a regular polyploid plate is very low, it seems most likely that the chromosomally unbalanced polyploid cells are more common than the well-balanced polyploid cells. A similar phenomenon was observed by Koller (1947) in human tumors. The giant cells with a very high number of chromosomes are occasionally observable; the chromosomes are usually small in size and lie

scattered in the cytoplasm. The number of chromosomes is so high that the degree of polyploidy of the cell cannot be determined. In the extreme case the cell with some 250 or more chromosomes was seen (Figs. 10, 37). All giant cells show abnormal orientation of metaphase chromosomes or the multipolar spindle. Division in the giant cells is almost arrested at metaphase and the chromosomes begin to disintegrate forming amorphous masses.

2. Mitotic abnormalities and the numerical changes of the chromosomes

Numerical change in the chromosome complement is an almost universal phenomenon in tumor cells. It has frequently been noted by many investigators in various human cancers, as well as in experimental tumors of rats and mice (cf. Makino's list, 1951). As described in the foregoing pages, various deviations of the chromosome number were observed also in the Yoshida sarcoma. It seems reasonable to offer here some comment on mitotic abnormalities of tumor cells which are to be regarded as direct or indirect causes of the numerical changes of chromosomes. For various types of mitotic abnormalities found in this sarcoma reference may be made to the previous paper (Makino and Yosida 1951).

It has been stated several times in the present paper that the tumor cells with the chromosome number of 40 or thereabouts, are the most frequent in this tumor, and that the variation of the chromosome number of tumor cells takes place around the number 40, there being a wide fluctuation ranging between ± 20 and ± 80. Furthermore, the cells containing chromosome numbers lower than ± 40 are more frequently found than those having a larger number than that. In these cases, as seen in Table 5, the fluctuation of the number is rather gradual. Such a gradual deviation of the chromosome number from the specific number (± 40) seems to be, in greater part, attributable to stickiness of chromosomes, chromosome bridges, lagging of chromosomes, or unequal distribution of chromosomes at anaphase. Stickiness of chromosomes usually form chromosome bridges at anaphase stretching between the poles (Figs. 11-14). Some chromosomes may be caught between the bridges, in which case they will mostly be excluded outside the nucleus. The fragmentation seems to occur in close association with the bridge-formation of sticky chromosomes. Often, a few chromosomes, two or three in number, lie extruded outside the nuclear palte or scattered in groups at an eccentric area on the equatorial plane (Fig. 14), due probably to the deficient or incomplete spindle formation. It may be these extruded elements that manifest themselves as the lagging chromosomes during anaphase. Or else, the laggards may comprise the chromosomes undergoing non-disjunction. Lagging elements to the number of 1 or 2 are most usual (Figs. 11, 27, 28), but there are cases that show 5, 6 or even 10 (Fig. 12). The size of laggards is also widely variable. The lagging chromosomes are either included in the separating chromosomes or fail to be taken into the daughter

nuclei. Unequal distribution of chromosomes at anaphase, or a migration of chromosomes in different numbers to poles, probably influences the variation of the chromosome number. This phenomenon by no means always concerns multipolarity or polyploidy ; it is abundantly seen in the bipolar spindle. Thus, stickiness, lagging of chromosomes and unequal distribution of chromosomes during anaphase may result in the production of nuclei in which some chromosomes are absent, or in which extra chromosomes are added to the specific number. Hence it follows that the gradual decrease or increase of the chromosome number at approximately the specific number 40 can be well explained by reference to the series of abnormalities such as above mentioned.

The most probable explanation for the sudden reduction of the chromosome number is connected with either the tripolar division or the elimination of some chromosomes at telophase. The tripolar spindle is not always restricted to polyploidy ; it is frequently seen in ordinary diploid cells. For instance, the results of observations made throughout the whole life span of a tumor rat, showed that 73 percent of the multipolar mitosis was tripolar, 22 percent was quadripolar and only 5 percent was pentapolar. Tripolar division results in different numbers of chromosoms at three poles. For instance, Figure 15 shows a tripolar division which had taken place in a probably subdiploid cell with ±40 chromosomes ; in one of the poles were found approximately the diploid number, while the other two poles showed about 27 and 11 elements, respectively. Figure 16 illustrates another example of a similar phenomenon ; three poles showed about 40, 20 and 15 chromosomes, respectively. Thus, the tripolar mitosis, when the division is completely finished, results in three cells with different chromosome numbers, and accordingly forms one of the causes for the sudden appearance of cells with greatly reduced chromosome numbers. The sudden decrease of the number may, on the other hand, possibly be explained by the elimination of some chromosomes lying off the equatorial plate. Very often, some chromosomes lie extruded outside the nuclear plate or scatter in a group at an eccentric area on the equatorial plane (Fig. 14). These unusual elements, when they are not few in number such as 10 or thereabouts, are often extruded outside the daughter nuclei at telophase and finally lost. Evidently, this results in the formation of nuclei in which for instance some 10 elements are absent.

There is evidence suggesting that the restitution-nucleus constitutes the origin of the subtetraploid cells. It is most likely that the fusion of two subdiploid nuclei with well-balanced ±40 chromosomes probably gives rise to the subtetraploid cell containing well-balanced chromosomes. This can strongly be supported by the occurrence of subtetraploid cells showing both a regular orientation of the metaphase chromosomes and a normal bipolar spindle. Figures 17 and 29 are probably cases being in process of fusion of two nuclei after separation to form a restitution-nucleus. The nucleus with subtriploid chromosomes can likewise

be well understood to arise through the fusion of two of three nuclei produced in the tripolar division, *i. e.*, by the association of two nuclei one of which contains subdiploid chromosomes and the other a reduced number of chromosomes as shown in Figure 15. But, no direct evidence has been met with as yet to support this interpretation.

The duplication of chromosomes without spindle formation, namely the process of the so-called endomitosis resulting in chromosome multiplication within the nuclear membrane, has frequently been recognized as a possible mechanism of the formation of polyploid cells (Levine 1931, Geitler 1939, Biesele, Poyner & Painter 1942). This may also be applicable for explaining the formation of the subtriploid, subtetraploid or other hyperploid cells in the present case. But there has been no evidence of endomitosis in this sarcoma so far as the observation has been gone.

For the change of the nuclear contents, the multipolar divisions strikingly participate as direct or indirect causes. Generally, the multipolar divisions result in the production of either several nuclei with variable chromosome numbers in which case a multinucleate cell is often formed or a many-lobed restitution nucleus. Multipolarity is by no means always connected with polyploidy. The multipolar division takes place either in subdiploid cells and those with a reduced chromosome number, or in polyploid cells. The failure of synchronization in separation of chromosomes, which usually occurs in close association with partial breakdown of the spindle mechanism, may induce abnormal orientation of chromosomes and the latter leads to the formation of mutipolar or incomplete spindle. For example, Figures 18 and 31 show a tripolar division which results in the formation of three cells with different chromosome numbers. Figure 32 illustrates the same in which a binucleate cell and a mono-nucleate cell were formed. Figures 19 and 33 indicate a tetrapolar division illustrating three daughter cells, one of which contains two nuclei, while the other two each include a single nucleus. Figures 20 and 34 again show a tetrapolar division which results in the production of two cells, one of them having three nuclei and the other a single nucleus. In Figures 21 and 22 are depicted two cases of pentapolar division. Figure 21 (Figs. 35 and 36 also) illustrates the formation of two cells, one having four nuclei and the other a single nucleus, whereas in Figure 22 are seen three cells, two of them with a single nucleus respectively and the other with three nuclei. And further, most of the multipolar divisions contain lagging chromosomes, or often show unequal distribution of chromosomes to poles. Thus, the multipolar division evidently gives rise to prominent multinucleate cells, highly influencing the numerical change of chromosomes in cells. Successive duplication of the above processes may result in very complicated contents of the nucleus, such as forming a giant cell. It is probable that, when the cell has reached a certain size it would become inviable. In the giant multipolar metaphases, the chromosomes begin to disintegrate forming amorphous blocks. There is evidence which shows that the percentage of multipolar metaphases in the total is considerably

higher than that of multipolar anaphase. This seems to suggest that most of multipolar metaphases never reach the anaphase stage. A comparable feature was found by Timonen & Therman (1950) in human cancers.

By way of summary, it may be said that various mitotic abnormalities, which may concern the behavior of the chromosomes or the cytoplasm or both, largely play a significant role in the numerical changes of chromosomes in tumor cells, and almost all of them lead to degeneration and death of the cells. As already stated, this is probably due to the disturbance of the inner balance between the nucleus and cytoplasm.

Here, attention should be paid to the fact that the degeneration of tumor cells by no means always depends upon the violent change of the chromosome number or unbalanced polyploidy. There are many tumor cells which are undergoing disintegration at the stage of metaphase. The results of approximate counting of the chromosome number in these cells revealed that the cells with the chromosome number 30-40 undergo disintegration at a considerable rate (Table 7). Though nothing has been learned up to the present to explain this phenomenon, a view involving the natural death of tumor cells through karyolysis is very probable.

Table 7. Approximate chromosome numbers observed in tumor cells which are undergoing disintegration at metaphase.

Chrom. numbers	±15—±20	±21—±30	±31—±40	±41—±50	±51—±60	Total
Number of cells observed	34	103	47	11	2	197
%	17.2	52.3	23.9	5.6	1.0	100.0

Summary

The mitotic rate and the variation of the chromosome number were observed in the Yoshida sarcoma, based on the daily material which was obtained throughout the whole life span of the tumor rat in a transplant generation. On account of their high frequency and definite rate of division, it can be concluded that there is a group or a strain of tumor cells with well-balanced subdiploid chromosome number of 40 or thereabouts, and that they primarily contribute to the growth of the tumor.

The variation of the chromosome number takes place around the specific number ±40, there being a wide range of fluctuation from about 20 to approximately 80. On the whole, the cells having lower chromosome numbers than ±40 are more frequent than those with larger numbers than that.

Some critical remarks were offered on the mitotic abnormalities which significantly concern the numerical changes of chromosomes in tumor cells.

Some considerations were offered on the mitotic capacity in tumor cells with a reduced or increased chromosome number.

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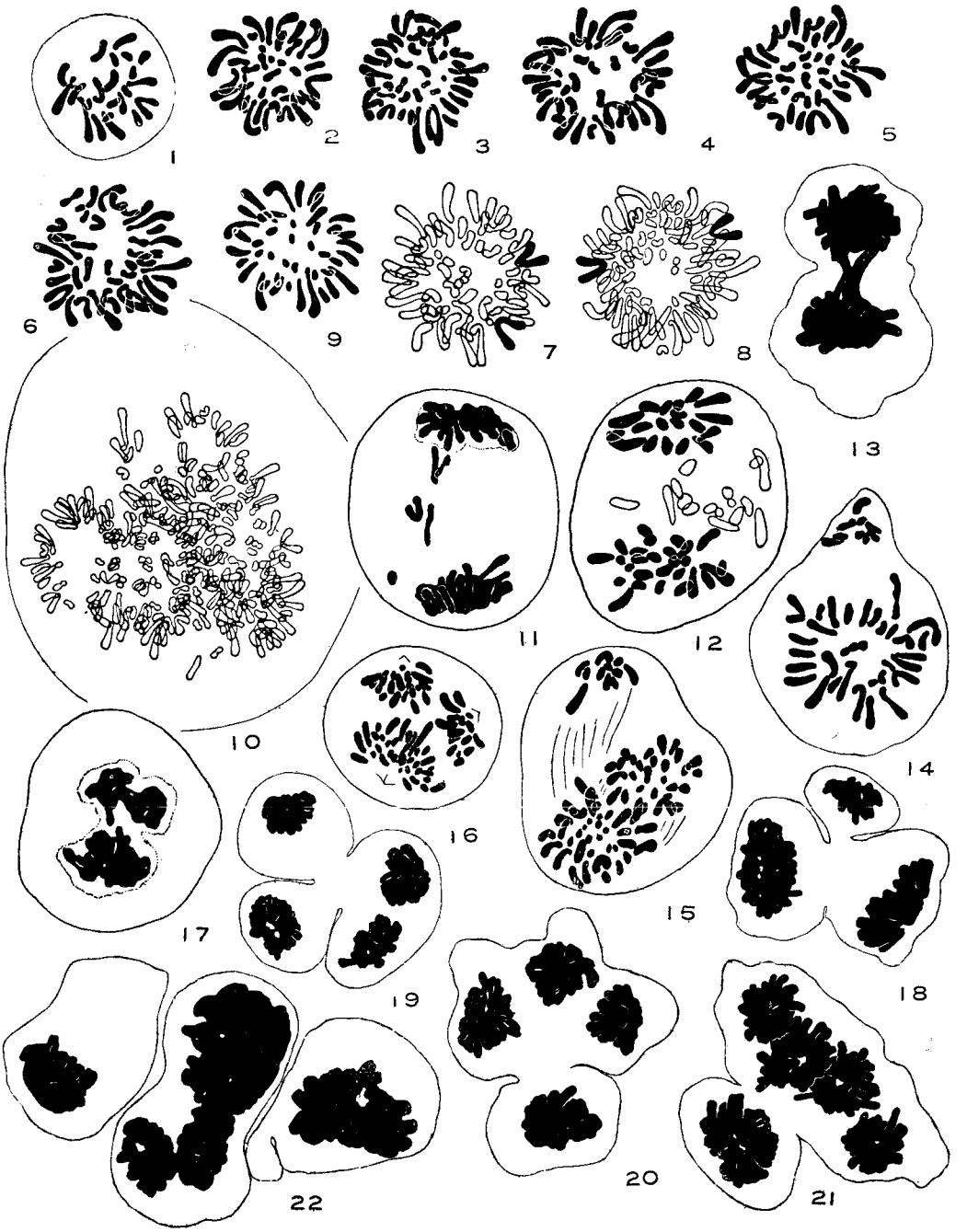
Explanation of Plate X

All are camera-lucida drawings of tumor cells; ca. ×1300, except Fig. 10, ca. ×800. **Fig. 1,** metaphase plate showing 22 chromosomes. **Figs. 2-6,** metaphase plates showing well-balanced subdiploid chromosomes; 37, 39, 40, 41 and 42 chromosomes in each. **Fig. 7,** subtriploid plate showing 60 chromosomes. **Fig. 8,** subtetraploid plate showing 80 chromosomes. **Fig. 9,** chromosomes of a blood cell, showing 42 elements. **Fig. 10,** metaphase plate of a giant cell, showing approximately 250 elements. **Figs. 11-12,** ana-telophases showing lagging chromosomes. **Fig. 13,** chromosome bridges at telophase. **Fig. 14,** showing some chromosomes extruded from the equatorial plate. **Fig. 15,** tripolar mitosis, showing about 40, 27 and 11 chromosomes at three poles. **Fig. 16,** the same, showing about 40, 15 and 25 chromosomes at three poles. **Fig. 17,** formation of restitution-nucleus. **Fig. 18,** tripolar telophase. **Fig. 19,** tetrapolar telophase, showing the formation of a bi-nucleate cell. **Fig. 20,** the same, showing the formation of a tri-nucleate cell. **Fig. 21,** pentapolar telophase, showing the formation of a tri-nucleate cell. **Fig. 22,** the same, showing the formation of a tri-nucleate cell.

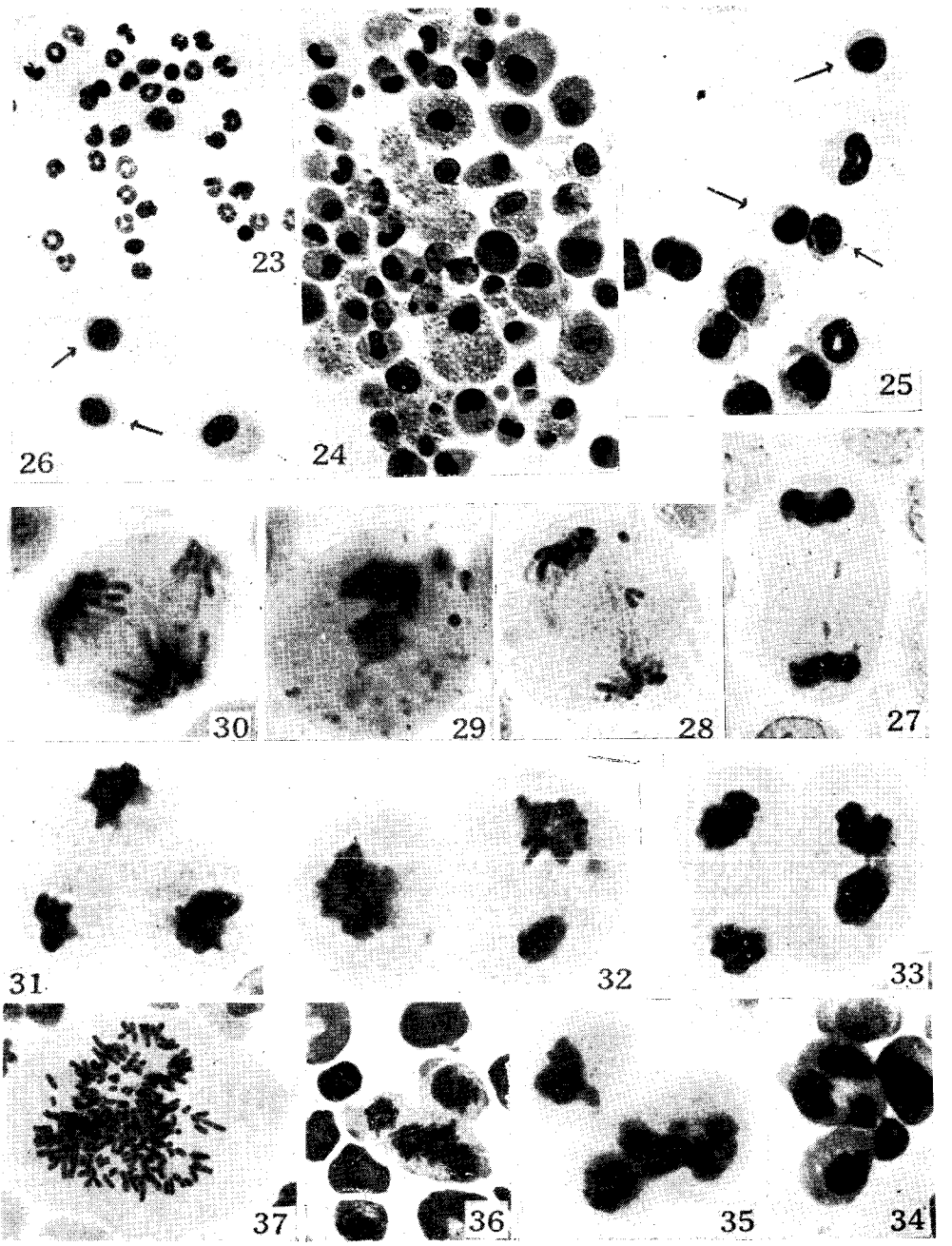
Explanation of Plate XI

All are photomicrographs of tumor cells.

Fig. 23, monocytes, lymphocytes, leucocytes, etc. in the normal ascites of the white rat. **Fig. 24,** showing the degeneration of tumor cells of large size found in the tumor ascites few hours after transplantation (acetocarmine). **Fig. 25,** showing two types of tumor cells, large and small, found in the tumor ascites about 20 hours after transplantation (Giemsa). **Fig. 26,** showing two types of tumor cells, large and small, in the tumor ascites just after the tumor-animal's death (acetocarmine). **Figs. 27-28,** showing lagging chromosomes at ana-telophase. **Fig. 29,** formation of restitution-nucleus. **Fig. 30,** tripolar telophase. **Fig. 31,** showing the formation of a bi-nucleate cell following the tripolar division. **Fig. 32,** the same. **Fig. 33,** tetrapolar division, showing the formation of a bi-nucleate cell. **Fig. 34,** tetrapolar division showing a tri-nucleate cell. **Fig. 35-36,** pentapolar divisions forming a tetra-nucleate cell. **Fig. 37,** metaphase plate of a giant cell, showing about 250 chromosomes. 23; ×400. 24-26, 34, 36, 37; ×600. 27-33, 35; ×1200. (*S. Makino photo.*)



Makino, S. & K. Kanô : Mitotic Frequency and Variation of Chromosome Number in the Yoshida Sarcoma.



Makino, S. & K. Kanô : Mitotic Frequency and Variation of Chromosome Number in the Yoshida Sarcoma.

Cytological Studies on Cancer

II. Daily Observations on the Mitotic Frequency and the Variation of the Chromosome Number in Tumor Cells of the Yoshida Sarcoma Through a Transplant Generation[1)]

By

Sajiro Makino and **Kyoko Kanô**

(Zoological Institute, Hokkaido University)

(With 37 Figures in 2 Plates)

The mechanism of the malignant growth of the tumor is a matter of fundamental importance as well as of primary concern in cancer research. Although surprising advance has recently been made in this field of cancer pathology as presented in a good many important reports, the state of knowledge still seems inadequate for clarifying many important problems growing up around this subject. A new approach to these fundamental problems, however, will be opened to a considerable extent by research in the field of cytology, especially by the study of chromosomes of the tumor ; much confusion underlaying these points has remained unsolved.

Based on the daily analysis of the frequency of mitotic abnormalities occurring in the Yoshida sarcoma, the findings reported in the previous study suggest that cells which are undergoing a regular division with well-balanced subdiploid chromosomes play a significant role in the growth of the tumor (Makino and Yosida 1951). The data presented in that paper were obviously scanty ; therefore, it was premature to attempt any conclusive statement concerning the phenomenon. The present study was undertaken with a view to further confirm the previously reported evidence, through close observations of the mitotic rate of tumor cells. Observations were carried out in close connection with investigations of the chromosome constitution in the Yoshida sarcoma.

The hereunder described observations were made using smear preparations

1) Contribution No. 255 from the Zoological Institute, Faculty of Science, Hokkaido University, Sapporo, Japan.

daily prepared according to the procedure as follows : The smear was made from a droplet of the tumor ascites once a day from a certain tumor rat through its whole life, starting with the first day after transplantation up to the last day at intervals of about 24 hours. The slides thus prepared were stained after both Giemsa's and acetocarmine methods. Accurate observations concerning chromosomes were made exclusively in the acetocarmine preparations. The present study was based on material derived from tumors transplanted in three different individuals of white rats belonging to the purely bred Wistar strain.

The authors' cordial thanks are due to Mr. Tosihide H. Yosida and Mr. Tatsuya Tanaka for their kind assistance in the course of this study. This paper constitutes a part of studies carried out with the aid of grants from the 'Kagaku-kenkyu Fund' and also the 'Kagakushiken-kenkyu Fund', both out of the Ministry of Education.

General course of the growth of the Yoshida sarcoma in a transplant generation

By intraperitoneal introduction the ascites sarcoma grows in the new host and leads the diseased animal to death in 12 days on an average. General course of the growth of the tumor is as follows :

The tumor ascites inoculated in the new host in successive transplantations contains two types of tumor cells (Fig. 25). The one comprises relatively large cells generally characterized by a considerable amount of cytoplasm and a large nucleus of bilobed or kidney shape, or sometimes of very complicated form. Giant cells containing huge nuclei of bizarre outline are of frequent occurrence in this type. The other comprises small cells which are remarkable for their very small amonut of cytoplasm and a rather spherical, well-defined nucleus of compact appearance (Fig. 25) ; both the cytoplasm and nucleus are intensely basophilic in staining.

Following the transplantation of the tumor ascites, a great many tumor cells introduced into the peritoneal cavity of the new host seem to undergo degeneration, since the cells in the course of disintegration and those showing various abnormalities appear at a high rate (Fig. 24). The dividing cells are very few. The majority of these degenerating cells are large in size. The mitotic figures of tumor cells make their gradual appearance about 24 hours after transplantation. The number of dividing cells progressively increases with the passage of time. On the 3rd or 4th day after transplantation, the peritoneal fluid of the host seems to be in the most favorable state for proliferation of tumor cells and the latter proliferate very rapidly under this circumstance. Towards the middle part of the life span of the tumor animal, that is, on the 5th or 6th day the cells undergoing mitosis are seen to be most numerous. Then, they begin gradually to decrease with time towards the latter part of the life span. In correlation to the decrease of the

dividing cells, cells showing mitotic abnormalities and disintegration increase in frequency. The tumor ascites proportionally shows a remarkable accumulation. Nearing the last day of the host animal's life, dividing cells suddenly descrease in number. In striking contrast, the cells showing abnormalities as well as those of disintegration appear at a remarkably high rate. By this time the accumulation of the tumor ascites reaches an enormous amount with a great expansion of the abdomen of the tumor animal. Mingled with these degenerating cells, there occur in the tumor ascites a number of resting cells, which are characterized by their small size containing a small amount of cytoplasm and well-defined, basophilic nuclei (Fig. 26). The malignant growth of the tumor having reached this stage leads to the death of the host animal.

Observations

1. Rate of mitosis in a transplant generation

The rate of mitosis in tumor cells was observed with the daily material through the whole life span of certain tumor rats. For convenience of description, the entire life span of the tumor-bearing animal was divided into three parts, namely the early, middle and latter parts. Viewing the whole material the mitotic frequency in tumor cells is seen to show a slight variation day by day, and also according to individuals. But, there is observable a characteristic tendency in tumor cells of this sarcoma with respect to the mitotic rate.

The specimen here to be considered died on the 16th day after transplantation of the tumor. Two thousand tumor cells were observed every day through the entire period of the life of the tumor-bearing animal, and the daily frequencly of dividing cells covering those of late prophase, metaphase, anaphase and telophase, was calculated in percentage on the basis of the above data. The results are given in Table 1, and graphed in Chart 1.

Referring to the data presented in both Table 1 and Chart 1, it is apparent that the number of dividing cells shows a gradual increase from the early part continuing towards the middle part of the life span. The rate of mitosis forms a peak curve through the middle part, decreasing gradually towards the latter part.

Table 1. Daily frequency of mitotic cells in a tumor rat. 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	13	14	15	16
% of dividing cells	1.7	4.4	4.6	3.8	4.0	4.4	3.9	3.2	3.0	3.3	3.2	1.9	0.5	0.4	0.8	0.2

Another example likewise represents a similar result, described in the paper of Kanô (1951).

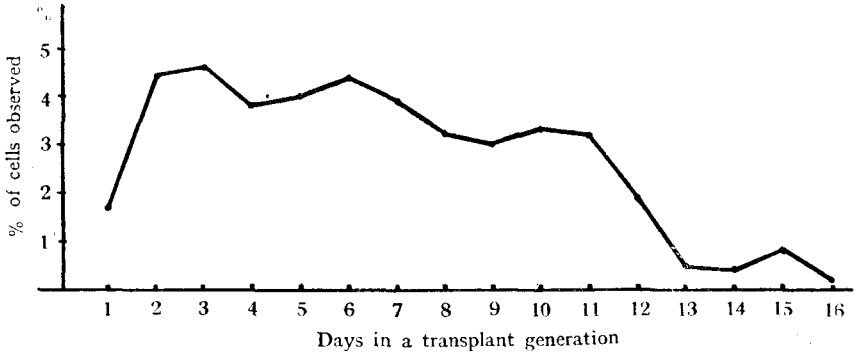


Chart 1. Graphical representation of the daily frequency of mitotic cells in a transplant generation of the tumor rat, based on the data in Table 1.

In the next observations based on the same material as above, attention was paid to the daily frequency of the cells with well-balanced subdiploid chromosomes having an ordinary metaphase configuration. The daily frequencies of the regularly dividing cells at metaphase, together with those of abnormal metaphase cells, were calculated on the basis of the total number of metaphase cells in the

Table 2. Daily frequency of the cells at metaphase, based on the same material as given in Table 1. The percentage was calculated on the basis of the total number of metaphase cells observed every day.

Days after transplantation	Regular cells at metaphase		Irregular cells at metaphase		Disintegrating cells at mataphase		Total number of metaphase cells observed	
	Number	%	Number	%	Number	%	Number	%
1	18	9.0	60	30.0	122	61.0	200	100.0
2	92	38 3	124	51.7	24	10.0	240	100.0
3	86	35.8	125	52.1	29	12.1	240	100.0
4	79	35.6	111	50.0	32	14.4	222	100 0
5	80	35.6	100	44.4	45	20.0	225	100.0
6	104	40.5	106	41.2	47	18.3	257	100.0
7	57	30.8	75	40.6	53	28.6	185	100.0
8	40	29.0	42	3.04	56	40.6	138	100.0
9	65	31.7	104	50.7	36	17.6	205	100.0
10	90	32 3	127	45.7	61	22.0	278	100.0
11	51	23.6	64	30.2	98	46.2	212	100.0
12 ≀ 16	15	8.5	71	42.5	81	45.5	167	100.0

daily observation. The results are given in Table 2.[1]

The data presented in Table 2 indicate that, on the first day after transplantation of the tumor the number of cells with well-balanced subdiploid chromosomes is extremely small, whereas cells showing abnormalities appear in a considerably large number. The frequency of cells with well-balanced chromosomes shows a remarkable increase with time, and during the middle part of the life span, namely on the 5th to 7th day after transplantation, they are found in an enormous number. It is by this time that the most active growth of the tumor is attained, with a pronounced increase of the tumor ascites. Towards the latter part of the animal's life, the chromosomally well-balanced cells show a gradual decrease in number, being replaced by cells showing mitotic abnormalities which show a gradual increase in number. On the 11th day, or later, after transplantation, the cells showing

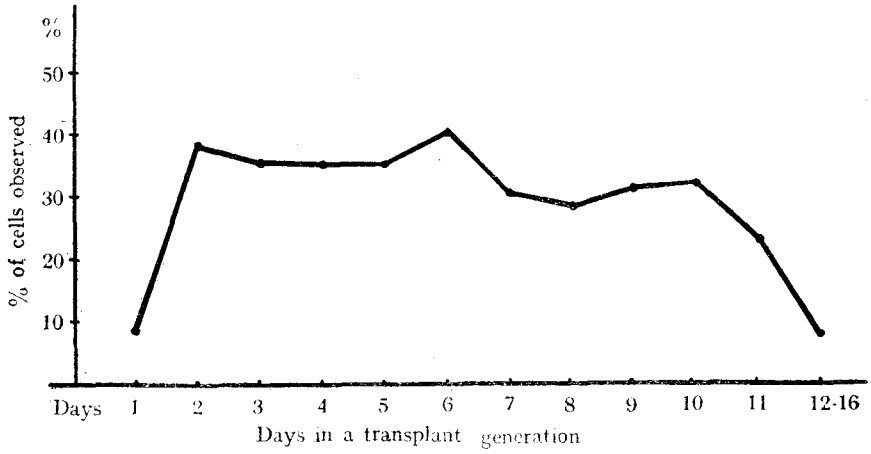


Chart 2. Graphical representation of the daily frequency of regularly dividing cells at metaphase, observed through a transplant generation, based on the data in Table 2. From the same material as used in Chart 1.

stickiness or coalescence of chromosomes, or disorganization of chromosomes appear at a strikingly high rate, in remarkable contrast to the low frequency of cells with subdiploid chromosomes.

1) In Table 2, the metaphase cells were classified into three types. Regular metaphase cells include the cells with a well-balanced subdiploid chromosomes, showing a regular feature and equatorial arrangement. The cells showing abnormal arrangement and aberrant number of chromosomes are designated as irregular metaphase cells. The disintegrating cells are those showing stickiness and coalescence of chromosomes, or disorganization of chromosomes at metaphase.

Table 1 indicated that the number of dividing cells shows a gradual increase from the early part continuing towards the middle part of the life span of the tumor animal, and decreases gradually towards the latter part. The data presented in Table 2 run parallel to the above ; that is to say, the frequency of chromosomally well-balanced subdiploid cells shows again a gradual increase from the early part, being high during the middle part, and decreases gradually towards the latter part of the life span. In other words, along with the increase of dividing cells, the frequency of cells with subdiploid chromosomes also increases, and the decreases of the former again responds to the decrease of the latter cells. This is well understood by reference to Charts 1 and 2, in which are found two curves running nearly parallel. Obviously, the cells showing mitotic abnormalities are unable to continue active multiplication and therefore they cannot affect the growth of the tumor. Thus, from the comparison of the data presented in Table 1 (and Chart 1) and Table 2 (and Chart 2), derived from the same material, the conclusion may be drawn that, among the dividing tumor cells, those with well-balanced subdiploid chromosomes play a significant role in the multiplication of cells in the growth of the tumor. To state the point otherwise, the multiplication of tumor cells in the new host in successive transplant generations is primarily attributable to the chromosomally well-balanced subdiploid cells. Obviously, these well-balanced cells show nothing unusual in either equatorial arrangement or constitution of chromosomes, and are quite regular in behavior just as occurs in normal cells.[1] Thus, the chromosomally well-balanced cells which are apparantly regular in respect to their chromosomes and in their behavior during mitosis contribute primarily to the growth of the tumor. Eventually, the results of the present observations agree with those of the previous study concerning the daily frequencies of mitotic abnormalities in this sarcoma.

2. Accounts on the chromosome number

In order to learn the chromosome number of the cells with well-balanced chromosomes having an ordinary metaphase configuration, which primarily are concerned with the growth of the tumor as above mentioned, the counting of the chromosomes was undertaken using the same preparations as employed in the above studies. Taking into consideration only the chromosomally well-balanced subdiploid cells with ordinary constitution and equatorial arrangement of chromosomes, the number of chromosomes was observed in the Giemsa-preparations, prepared every day through the whole life span of a tumor rat. The Giemsa-preparations, however, yielded no difinite results as for the chromosome number, so that only an approximate estimation of the number, such as 30-40, was possible in this case.

1) In this respect, see the next paper (Makino 1952).

The results of observations are shown in Table 3. It is evident from this table, that in the cells with a well-balanced metaphase configuration the chromosome number shows a small range of variation, and that cells with the chromosome numbers ranging from 30 to 40 are the most frequent of all. The data here obtained were confirmed by those coming from another specimen which likewise showed the same result, as given in Table 4. (In this case the chromosome counting was made

Table 3. Daily observations of the chromosome numbers in tumor cells through a transplant generation, from the same specimen as given in Tables 1 and 2. (Giemsa-preparations).

Days after transplantation	Chrom. Numbers				Total
	20—33	31—40	41—50	51—60	
	Number of cells observed				
2	12	18	1		31
3	5	21	4		30
4	7	18	4		29
5	13	29	1		43
6	7	23	6	1	37
7	6	15	2	1	24
8	10	13	2		25
9	9	19	10	2	40
10	9	21	8	2	40
11	10	22	3		35
12					
16	5	10			15
Total	93	209	41	6	349
%	26.6	60.0	11.7	1.7	100.0

Table 4. Chromosome numbers observed in a sample taken on the 5th day after transplantation of the tumor.

	Chromosome numbers			Total
	20—30	31—40	41—45	
Number of cells observed	7	63	10	80
%	8.75	78.75	12.5	100.0

in a sample taken on the 5th day after transplantation of the tumor in which dividing cells showed the highest frequency). Study of Table 4 indicates that the cells having 30-40 chromosomes accounted for 78.75% in a total of 80 metaphase cells under observation. Based on the above facts, it may be concluded that the majority of the chromosomally well-balanced cells contain 30-40 chromosomes,

so far as the rough counting based on Giemsa-preparations is concerned. Therefore, it can be said that probably the cells which primarily contribute to the growth of the tumor have the chromosome numbers ranging from 30 to 40.

Now, the necessity arises in the next step to get as accurate as possible counts of the chromosome number in tumor cells. However, the Giemsa-preparations are not useful at all for the exact study of chromosomes, because they yield some artifacts such as temporary fusion or abnormal swelling of chromosomes which all hamper close observation to a great extent. The acetocarmine smear method, on the contrary, proved to be excellent for the study of chromosomes, since by this method the chromosomes at metaphase are visible with a well-defined sharp outline showing no sign of irregular agglutination. The following observations were carried out with acetocarmine smear preparations exclusively. They were prepared every day with the tumor ascites secured from a new specimen different from that used in the above observations. This rat died on the 12th day after transplantation of the tumor. In observation, only clear metaphase cells were selected in which the adequate counting of the chromosome number is possible to a considerable extent. The daily variations of the chromosome numbers were observed through 12 days, from the first day after transplantation of the tumor up to the last day of life. The giant cells were disregarded. Even in good plates, an uncertainty of ± 1 could not be avoided in counting, except in very excellent ones. The results of this survey are arranged in Table 5. Referring to this table it is apparent that the number of chromosomes of tumor cells varies with a rather wide range : between about 22 for the smallest extremity and 80 for the largest one (Figs. 1-8). The variation of the number is rather gradational around a figure close to 40. On the whole, however, cells having chromosomes numbering less than 42 are more frequent than those numbering more. The latter cells are rather

Table 5. Daily observations on the chromosome numbers in tumor cells through a

Chrom. No. / Days	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
1				1					3			4	1	5	1		2		2	2	4	1		2				
2					1	2	2	2	3		5	6	7	7	9	7	6	5	8	6	5	1	2	1	1				
3	1	1							2	2	2	3	4	4	3	5	5	6	3	4	3	1							
4					1				2	4	5	4	3	2	6	7	5	5	7	5	1				1				
5							1		1		2	1	1	4	4	6	3	6	8	6	3	1	2						
6							1	1	2	1	2	2	2	2	5	4	4	6	2	5	3	1	1		1				
7						1						2		4	6	5	8	5	8	3	4	1		1					
8									1		1	1	1	1	1	3	8	6	9	11	5	1							
9										1			2	3	5	3	4	11	8	8	3								
10											2	2	1	3	5	5	6	8	7	4	3								1
11											1	1			2	3	4	6	12	11	4	2	2				1		
12											1	1	3	3	4	5	5	4	5	7	1								1
Total	1	0	1	1	2	3	4	3	14	8	21	27	25	38	51	53	60	68	79	72	39	9	7	4	3	0	1	0	2

infrequent. Also, true polyploidy seems to be very rare in occurrence or absent. But there appear very rarely subtriploidy showing ±60 (Fig. 7) and subtetraploidy having ±80 (Fig. 8). Special attention should be paid to the fact that the tumor cells showing chromosome numbers which range from ±35 to ±42 are very frequent for each day. Particularly, the cells with 38, 39, 40 and 41 chromosomes appear each day at a strikingly high rate (Figs. 2-6). Among them, cells having 40 chromosomes are the most frequent, and those with 41 chromosomes rank second. This fact is important in that most frequently the tumor cells contain the chromosomes very nearly approximate in number to those of the host, *Rattus norvegicus*, in which 42 diploid chromosomes were found in both germ and somatic cells (Makino 1942, 1943, Tanaka 1951). It is also remarkable that cells with a lower chromosome number than ±40 are more frequent than those with larger number than ±40.

In order to learn the daily frequency of the cells with the chromosome numbers 38-41, the necessary data were picked out from Table 5 and arranged in Table 6. In this table, the total sum of cells with 38-41 chromosomes is given for each day, and the percentage is calculated on the basis of the total number of cells observed. Since the data presented in Talbe 6 are based on the clear metaphase cells adequate for chromosome counting alone, it is not permissible to use them as a criterion for comparison with those of Table 3. The high percentage of the cells with 38-41 chromosomes during the latter part of the life span of the tumor rat as given in Table 6 is undoubtedly attributable to the absence of data concerning giant cells or cells undergoing disintegration. By comparison of Table 3 with Table 4, it is allowable to say that the daily occurrence of cells with 30-40 chromosomes is nearly in a parallel relation to that of cells with 38-41 chromosomes. This implies that the cells with the chromosome numbers between 30 and 40 (Table 3) may be largely replaced, if not entirely, with those which contain approximately 40 chromo-

transplant generation of the Yoshida sarcoma, based on acetocarmine preparations.

51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	Total
1				1																										30
	1					1										1			1											90
		1																												50
					1				1																					60
																			1											50
					1					1						1							1						1	50
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																					1				1					50
									1				1								1									50
			1																											50
																														40
0	1	1	2	1	2	1	0	0	2	1	0	0	1	0	1	1	1	0	2	0	1	2	2	1	1	0	0	0	1	620

somes. From this conception, and by reference again to the data of Tables 1 and 2, it follows that the tumor cells which are characterized by a well-defined, regular metaphase configuration, and which play a significant part in the growth of the tumor, have the chromosome numbers of approximately 40.

Table 6. Picked-out data from Table 5, showing a daily frequency of the cells with 38—41 chromosomes.

Days after transplantation	No. of cells with 38—41 chroms. and their percentage		Total of cells observed
1	6	20.0%	30
2	25	27.8	90
3	18	36.0	50
4	22	36.6	60
5	23	46.0	50
6	17	34.0	50
7	24	48.0	50
8	34	68.0	50
9	31	62.0	50
10	25	50.0	50
11	33	66.0	50
12	21	52.0	40
Total	279	45.0	620

Summing up, it may be said that, on account of their high frequency and a definite rate of division, the tumor cells with 40 chromosomes or thereabouts, primarily contribute to the growth of the tumor through their active proliferation. And further, the variation of the chromosome number takes place arround the number 40, showing a wide fluctuation from about 20 to approximately 80. As a whole the cells containing chromosome numbers lower than ±40 are more remarkably frequent than those with numbers larger than ±40.

Now, our particular interests concern the chromosome constitution of the cells which contain a well-balanced subdiploid set of chromosomes, 40 or thereabouts in number, and are responsible for the growth of the tumor. Observations by the senior author (S. M.) revealed that these cells carry a quite characteristic karyotype which is of this sarcome proper and again strikingly differentiated from that of the host, *Ratlus norvegicus* (Fig. 9), being characterized by a certain number of V-shaped elements (Figs. 2-6). Details will be given in the next paper of these serial studies.

Remarks

1. On the mitotic capacity in cells with reduced or increased chromosome numbers

It was pointed out in the foregoing observations that there is a group or

strain of cells which contain the chromosome numbers of 40 or thereabouts, and further that they are primarily responsible for the growth of the tumor on account of their strikingly high frequency and definite mitotic rate. It is of interest that the specific number ±40 here concerned is closely approximate to the normal chromosome number of the host in which the diploid number of 42 has been well established. Winge (1930) reported in the tar-carcinoma of mice that the cells with 36-40 chromosomes were most frequent (the normal number for mice being 40).

As already mentioned, the variation of the chromosome number in this sarcoma takes place around the specific number ±40. But, noticeably the cells with lower numbers than ±40 show larger frequency than those with higher numbers than ±40 (cf. Table 5). Particularly the cells having 36, 37, 38, 39, 40 and 41 chromosomes show a strongly increased frequency. This evidence is an indication that the cells with such chromosome numbers have a mitotic capacity exceeding that of other cells. Until recently, it has been considered that a nucleus with the complete chromosome number is necessary of the regular functioning of cells (Koller 1943, 1947), and that cells with deficient nuclei can survive only under very specific conditions (Barber 1941, Sax 1942), the life of such cells always being short (Koller & Smithers 1946). The Yoshida sarcoma here under consideration furnishes, however, an apposite example, since cells with reduced or increased chromosome number are still able to divide and to continue dividing. Similar evidence was presented by Koller (1947) in the human adenocarcinoma. These facts seem to imply that, because the cells remain active in spite of their deficient nuclei, the complete chromosome set is not necessarily required for the accomplishment of normal cell behavior. Koller (1947) interpreted the phenomenon by considering that mitotic activity is under cytoplasmic and not nuclear control. However, this assumption seems not adequate for the present case. The present authors are of opinion that the inner balance held between the nucleus and cytoplasm is necessary for the regular functioning of cells, namely that under the balanced condition the nucleus can control the cytoplasm to accomplish normal cell function, due probably to the co-operation between the former and the latter. So long as the inner balance between the nucleus and cytoplasm remains favourable, the cells have mitotic capacity. It appears likely that the normal nucleic acid cycle of the chromosomes can proceed, or remain active, within this limit. If the nucleus contain a certain set of chromosomes necessary for controlling the cytoplasm, or else for co-operation with the latter, it should permit mitotic activity without difficulty, even though the chromosome set is not always entirely complete. The case of the Yoshida sarcoma is an example strongly favouring this assumption. Because of their highest mitotic rate, as seen in Table 5, the cells with 40 chromosomes might be in the most well-balanced condition in the nucleus-cytoplasmic relationship. The frequency of the cells with chromosome numbers lower than 40, and that of the cells with higher numbers shows a gradual decrease, as the chromosome

number becomes either greater or less than 40. Probably, the inner balance of cells might increasingly be disturbed and therefore mitotic activity might progressively be interrupted, as the number varies above or below 40.

Koller (1947) further claimed that tumor cells may be dependent on each other in many respects, and that in undergoing mitosis, a specific substance necessary for chromosome synthesis is transferred from one cell to adjacent cells. For this reason, according to him, cells with a reduced chromosome number are able to divide. Also, there is another but similar view that nuclei with normal chromosome sets are capable of controlling the mitosis of other adjacent nuclei which lack a normal chromosome set (Svärdson 1945). These are interseting ideas for the interpretation of the phenomenon respectively, but they cannot be applicable for the present case, because the Yoshida sarcoma is a fluid tumor in which tumor cells always occur independently from one another in a form of a suspension without forming massive tissue.

Here is also another view involving a hypothesis concerning mitotic genes; namely, there are in the nucleus a series of genes which are required for the accomplishment of normal mitosis (cf. Svärdson 1945). The mitotic genes may either be concentrated to a few chromosomes or else each chromosome may have a set of mitotic genes. This assumption cannot be easily repudiated, since it must be of considerable importance in consideration of mitotic activity of cells with a reduced chromosome number. However, knowledge of these mitotic genes is exceedingly deficient. As yet very little is known about the various fields of activity of these genes (Mather 1942). Though nothing can be said at present, this hypothesis is also of considerable interest in connection with the theory of gene or chromosome mutation as connected with the origin of cancer.

As is obvious by reference to Table 5, the occurrence of a true polyploidy seems to be very rare or absent in tumor cells. But, the cells with subtriploid chromosome numbers such as ± 55 and ± 60, and those with subtetraploid numbers such as ± 75 and ± 80, are rarely found (Figs. 7-8). In the majority of polyploid cells, abnormal orientation of metaphase chromosomes and the formation of multipolar or incomplete spindle occurred very often (cf. the column of irregular metaphase cells in Table 2), so that the formation of a regular metaphase plate is interrupted. Of course, these obstruct a regular proceeding of cell division and lead to death of the cells, due to the numerous cytological abnormalities induced. Only the cells with a well-balanced chromosome number must form a regular metaphse plate, showing a normal orientation and arrangement of chromosomes. On account of the fact that the frequency of a regular polyploid plate is very low, it seems most likely that the chromosomally unbalanced polyploid cells are more common than the well-balanced polyploid cells. A similar phenomenon was observed by Koller (1947) in human tumors. The giant cells with a very high number of chromosomes are occasionally observable; the chromosomes are usually small in size and lie

scattered in the cytoplasm. The number of chromosomes is so high that the degree of polyploidy of the cell cannot be determined. In the extreme case the cell with some 250 or more chromosomes was seen (Figs. 10, 37). All giant cells show abnormal orientation of metaphase chromosomes or the multipolar spindle. Division in the giant cells is almost arrested at metaphase and the chromosomes begin to disintegrate forming amorphous masses.

2. Mitotic abnormalities and the numerical changes of the chromosomes

Numerical change in the chromosome complement is an almost universal phenomenon in tumor cells. It has frequently been noted by many investigators in various human cancers, as well as in experimental tumors of rats and mice (cf. Makino's list, 1951). As described in the foregoing pages, various deviations of the chromosome number were observed also in the Yoshida sarcoma. It seems reasonable to offer here some comment on mitotic abnormalities of tumor cells which are to be regarded as direct or indirect causes of the numerical changes of chromosomes. For various types of mitotic abnormalities found in this sarcoma reference may be made to the previous paper (Makino and Yosida 1951).

It has been stated several times in the present paper that the tumor cells with the chromosome number of 40 or thereabouts, are the most frequent in this tumor, and that the variation of the chromosome number of tumor cells takes place around the number 40, there being a wide fluctuation ranging between ± 20 and ± 80. Furthermore, the cells containing chromosome numbers lower than ± 40 are more frequently found than those having a larger number than that. In these cases, as seen in Table 5, the fluctuation of the number is rather gradual. Such a gradual deviation of the chromosome number from the specific number (± 40) seems to be, in greater part, attributable to stickiness of chromosomes, chromosome bridges, lagging of chromosomes, or unequal distribution of chromosomes at anaphase. Stickiness of chromosomes usually form chromosome bridges at anaphase stretching between the poles (Figs. 11-14). Some chromosomes may be caught between the bridges, in which case they will mostly be excluded outside the nucleus. The fragmentation seems to occur in close association with the bridge-formation of sticky chromosomes. Often, a few chromosomes, two or three in number, lie extruded outside the nuclear palte or scattered in groups at an eccentric area on the equatorial plane (Fig. 14), due probably to the deficient or incomplete spindle formation. It may be these extruded elements that manifest themselves as the lagging chromosomes during anaphase. Or else, the laggards may comprise the chromosomes undergoing non-disjunction. Lagging elements to the number of 1 or 2 are most usual (Figs. 11, 27, 28), but there are cases that show 5, 6 or even 10 (Fig. 12). The size of laggards is also widely variable. The lagging chromosomes are either included in the separating chromosomes or fail to be taken into the daughter

nuclei. Unequal distribution of chromosomes at anaphase, or a migration of chromosomes in different numbers to poles, probably influences the variation of the chromosome number. This phenomenon by no means always concerns multipolarity or polyploidy ; it is abundantly seen in the bipolar spindle. Thus, stickiness, lagging of chromosomes and unequal distribution of chromosomes during anaphase may result in the production of nuclei in which some chromosomes are absent, or in which extra chromosomes are added to the specific number. Hence it follows that the gradual decrease or increase of the chromosome number at approximately the specific number 40 can be well explained by reference to the series of abnormalities such as above mentioned.

The most probable explanation for the sudden reduction of the chromosome number is connected with either the tripolar division or the elimination of some chromosomes at telophase. The tripolar spindle is not always restricted to polyploidy ; it is frequently seen in ordinary diploid cells. For instance, the results of observations made throughout the whole life span of a tumor rat, showed that 73 percent of the multipolar mitosis was tripolar, 22 percent was quadripolar and only 5 percent was pentapolar. Tripolar division results in different numbers of chromosoms at three poles. For instance, Figure 15 shows a tripolar division which had taken place in a probably subdiploid cell with ±40 chromosomes ; in one of the poles were found approximately the diploid number, while the other two poles showed about 27 and 11 elements, respectively. Figure 16 illustrates another example of a similar phenomenon ; three poles showed about 40, 20 and 15 chromosomes, respectively. Thus, the tripolar mitosis, when the division is completely finished, results in three cells with different chromosome numbers, and accordingly forms one of the causes for the sudden appearance of cells with greatly reduced chromosome numbers. The sudden decrease of the number may, on the other hand, possibly be explained by the elimination of some chromosomes lying off the equatorial plate. Very often, some chromosomes lie extruded outside the nuclear plate or scatter in a group at an eccentric area on the equatorial plane (Fig. 14). These unusual elements, when they are not few in number such as 10 or thereabouts, are often extruded outside the daughter nuclei at telophase and finally lost. Evidently, this results in the formation of nuclei in which for instance some 10 elements are absent.

There is evidence suggesting that the restitution-nucleus constitutes the origin of the subtetraploid cells. It is most likely that the fusion of two subdiploid nuclei with well-balanced ±40 chromosomes probably gives rise to the subtetraploid cell containing well-balanced chromosomes. This can strongly be supported by the occurrence of subtetraploid cells showing both a regular orientation of the metaphase chromosomes and a normal bipolar spindle. Figures 17 and 29 are probably cases being in process of fusion of two nuclei after separation to form a restitution-nucleus. The nucleus with subtriploid chromosomes can likewise

be well understood to arise through the fusion of two of three nuclei produced in the tripolar division, *i. e.*, by the association of two nuclei one of which contains subdiploid chromosomes and the other a reduced number of chromosomes as shown in Figure 15. But, no direct evidence has been met with as yet to support this interpretation.

The duplication of chromosomes without spindle formation, namely the process of the so-called endomitosis resulting in chromosome multiplication within the nuclear membrane, has frequently been recognized as a possible mechanism of the formation of polyploid cells (Levine 1931, Geitler 1939, Biesele, Poyner & Painter 1942). This may also be applicable for explaining the formation of the subtriploid, subtetraploid or other hyperploid cells in the present case. But there has been no evidence of endomitosis in this sarcoma so far as the observation has been gone.

For the change of the nuclear contents, the multipolar divisions strikingly participate as direct or indirect causes. Generally, the multipolar divisions result in the production of either several nuclei with variable chromosome numbers in which case a multinucleate cell is often formed or a many-lobed restitution nucleus. Multipolarity is by no means always connected with polyploidy. The multipolar division takes place either in subdiploid cells and those with a reduced chromosome number, or in polyploid cells. The failure of synchronization in separation of chromosomes, which usually occurs in close association with partial breakdown of the spindle mechanism, may induce abnormal orientation of chromosomes and the latter leads to the formation of mutipolar or incomplete spindle. For example, Figures 18 and 31 show a tripolar division which results in the formation of three cells with different chromosome numbers. Figure 32 illustrates the same in which a binucleate cell and a mono-nucleate cell were formed. Figures 19 and 33 indicate a tetrapolar division illustrating three daughter cells, one of which contains two nuclei, while the other two each include a single nucleus. Figures 20 and 34 again show a tetrapolar division which results in the production of two cells, one of them having three nuclei and the other a single nucleus. In Figures 21 and 22 are depicted two cases of pentapolar division. Figure 21 (Figs. 35 and 36 also) illustrates the formation of two cells, one having four nuclei and the other a single nucleus, whereas in Figure 22 are seen three cells, two of them with a single nucleus respectively and the other with three nuclei. And further, most of the multipolar divisions contain lagging chromosomes, or often show unequal distribution of chromosomes to poles. Thus, the multipolar division evidently gives rise to prominent multinucleate cells, highly influencing the numerical change of chromosomes in cells. Successive duplication of the above processes may result in very complicated contents of the nucleus, such as forming a giant cell. It is probable that, when the cell has reached a certain size it would become inviable. In the giant multipolar metaphases, the chromosomes begin to disintegrate forming amorphous blocks. There is evidence which shows that the percentage of multipolar metaphases in the total is considerably

higher than that of multipolar anaphase. This seems to suggest that most of multipolar metaphases never reach the anaphase stage. A comparable feature was found by Timonen & Therman (1950) in human cancers.

By way of summary, it may be said that various mitotic abnormalities, which may concern the behavior of the chromosomes or the cytoplasm or both, largely play a significant role in the numerical changes of chromosomes in tumor cells, and almost all of them lead to degeneration and death of the cells. As already stated, this is probably due to the disturbance of the inner balance between the nucleus and cytoplasm.

Here, attention should be paid to the fact that the degeneration of tumor cells by no means always depends upon the violent change of the chromosome number or unbalanced polyploidy. There are many tumor cells which are undergoing disintegration at the stage of metaphase. The results of approximate counting of the chromosome number in these cells revealed that the cells with the chromosome number 30-40 undergo disintegration at a considerable rate (Table 7). Though nothing has been learned up to the present to explain this phenomenon, a view involving the natural death of tumor cells through karyolysis is very probable.

Table 7. Approximate chromosome numbers observed in tumor cells which are undergoing disintegration at metaphase.

Chrom. numbers	±15—±20	±21—±30	±31—±40	±41—±50	±51—±60	Total
Number of cells observed	34	103	47	11	2	197
%	17.2	52.3	23.9	5.6	1.0	100.0

Summary

The mitotic rate and the variation of the chromosome number were observed in the Yoshida sarcoma, based on the daily material which was obtained throughout the whole life span of the tumor rat in a transplant generation. On account of their high frequency and definite rate of division, it can be concluded that there is a group or a strain of tumor cells with well-balanced subdiploid chromosome number of 40 or thereabouts, and that they primarily contribute to the growth of the tumor.

The variation of the chromosome number takes place around the specific number ±40, there being a wide range of fluctuation from about 20 to approximately 80. On the whole, the cells having lower chromosome numbers than ±40 are more frequent than those with larger numbers than that.

Some critical remarks were offered on the mitotic abnormalities which significantly concern the numerical changes of chromosomes in tumor cells.

Some considerations were offered on the mitotic capacity in tumor cells with a reduced or increased chromosome number.

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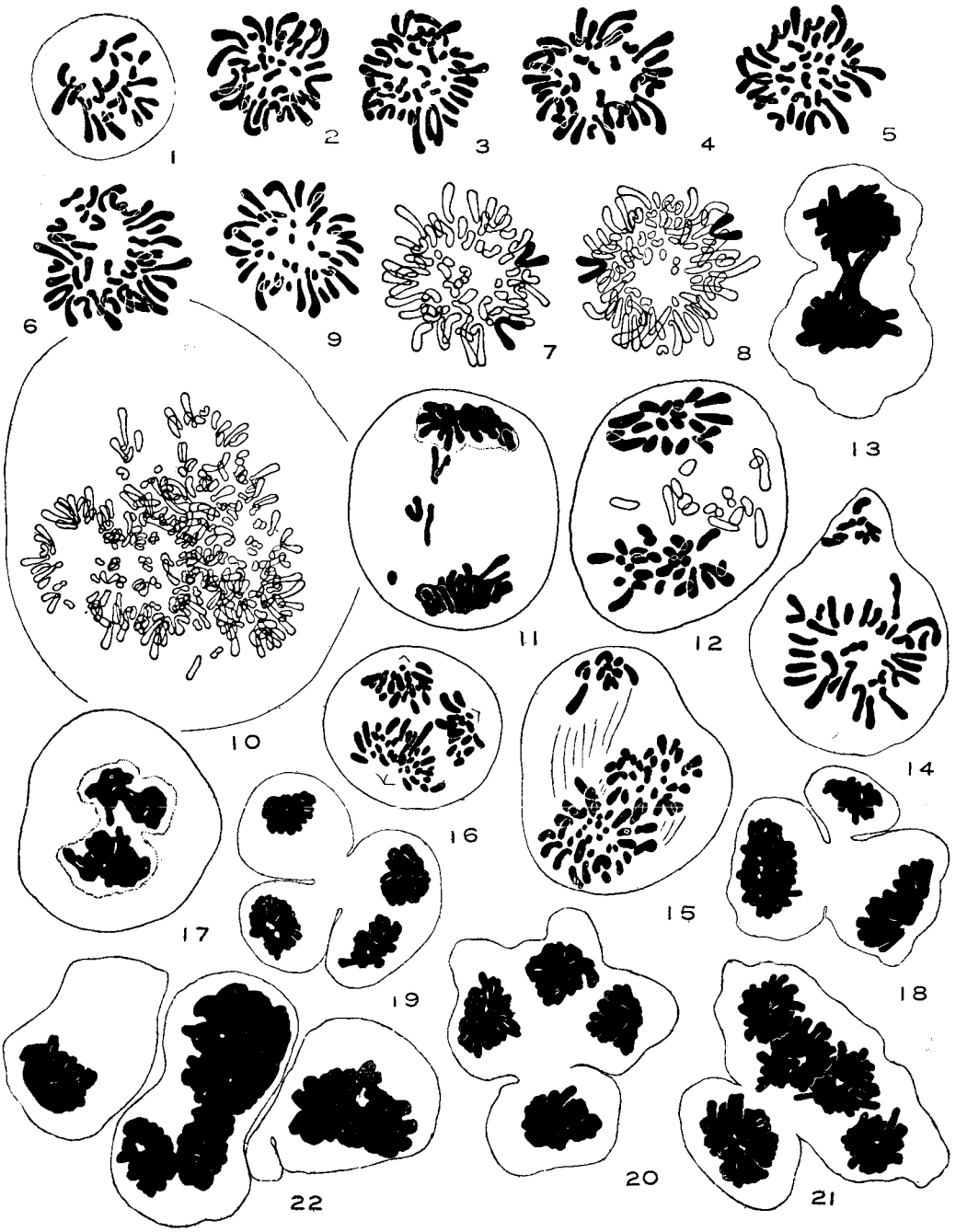
Explanation of Plate X

All are camera-lucida drawings of tumor cells; ca. ×1300, except Fig. 10, ca. ×800. **Fig. 1,** metaphase plate showing 22 chromosomes. **Figs. 2-6,** metaphase plates showing well-balanced subdiploid chromosomes; 37, 39, 40, 41 and 42 chromosomes in each. **Fig. 7,** subtriploid plate showing 60 chromosomes. **Fig. 8,** subtetraploid plate showing 80 chromosomes. **Fig. 9,** chromosomes of a blood cell, showing 42 elements. **Fig. 10,** metaphase plate of a giant cell, showing approximately 250 elements. **Figs. 11-12,** ana-telophases showing lagging chromosomes. **Fig. 13,** chromosome bridges at telophase. **Fig. 14,** showing some chromosomes extruded from the equatorial plate. **Fig. 15,** tripolar mitosis, showing about 40, 27 and 11 chromosomes at three poles. **Fig. 16,** the same, showing about 40, 15 and 25 chromosomes at three poles. **Fig. 17,** formation of restitution-nucleus. **Fig. 18,** tripolar telophase. **Fig. 19,** tetrapolar telophase, showing the formation of a bi-nucleate cell. **Fig. 20,** the same, showing the formation of a tri-nucleate cell. **Fig. 21,** pentapolar telophase, showing the formation of a tri-nucleate cell. **Fig. 22,** the same, showing the formation of a tri-nucleate cell.

Explanation of Plate XI

All are photomicrographs of tumor cells.

Fig. 23, monocytes, lymphocytes, leucocytes, etc. in the normal ascites of the white rat. **Fig. 24,** showing the degeneration of tumor cells of large size found in the tumor ascites few hours after transplantation (acetocarmine). **Fig. 25,** showing two types of tumor cells, large and small, found in the tumor ascites about 20 hours after transplantation (Giemsa). **Fig. 26,** showing two types of tumor cells, large and small, in the tumor ascites just after the tumor-animal's death (acetocarmine). **Figs. 27-28,** showing lagging chromosomes at ana-telophase. **Fig. 29,** formation of restitution-nucleus. **Fig. 30,** tripolar telophase. **Fig. 31,** showing the formation of a bi-nucleate cell following the tripolar division. **Fig. 32,** the same. **Fig. 33,** tetrapolar division, showing the formation of a bi-nucleate cell. **Fig. 34,** tetrapolar division showing a tri-nucleate cell. **Fig. 35-36,** pentapolar divisions forming a tetra-nucleate cell. **Fig. 37,** metaphase plate of a giant cell, showing about 250 chromosomes. 23; ×400. 24-26, 34, 36, 37; ×600. 27-33, 35; ×1200. (*S. Makino photo.*)



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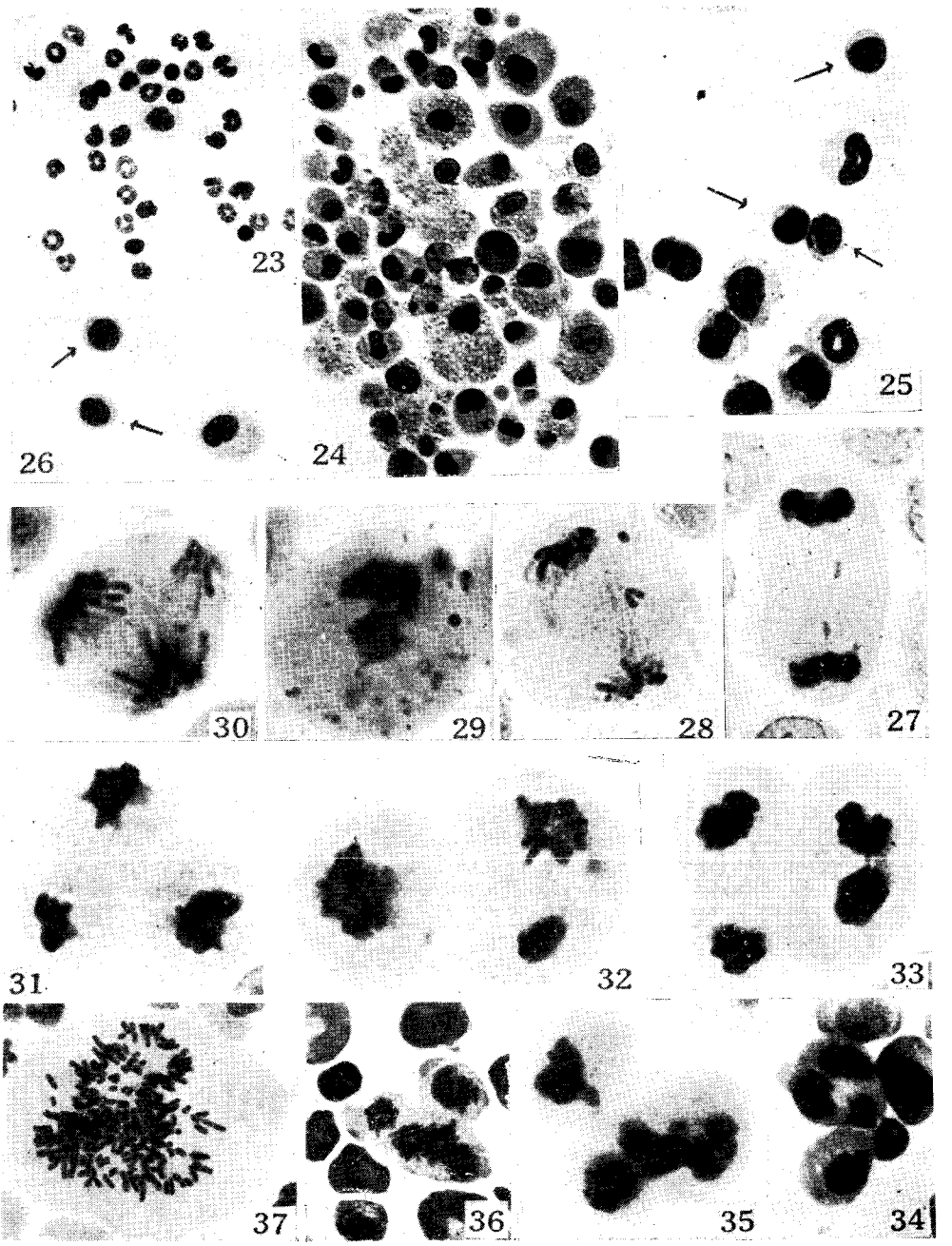

Makino, S. & K. Kanô : Mitotic Frequency and Variation of Chromosome Number in the Yoshida Sarcoma.