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EFFECT OF HETEROLOGOUS TRANSPLANTATION OF H 4-II-E RAT HEPATOMA CELLS INTO THE CHEEK POUCH OF IRRADIATED HAMSTER*

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ABSTRACT

A series of transplant-explant experiments using the H 4-II-E rat hepatoma cell line and golden hamsters have been carried out. The original tissue culture cells, which had a modal chromosome number of 51, implanted into the cheek pouches of irradiated hamster (400 rads) produced tumors which were typical hepatomas histologically. Cells from these tumors were placed back into tissue culture and were transferred twice more into the cheek pouches of irradiated animals and tissue culture. These were termed the first, second and third selection, respectively.

At each stage of selection, chromosome counts, karyotypes and histological and biological characteristics of the cells were determined. The explant from the first selection tumors and that from the second selection tumors were found to have 53 and 54 chromosome modes, respectively. Two explants derived from separate animals in the third stage of selection each showed different modes of chromosome number (53 and 54). The explants with a modal chromosome number of 54 had a more homogeneous karyotype than those with 53 chromosomes, and the former appeared to be less malignant than the latter, as judged by transplantability, growth rate of the cells in the hydrocortisone treated hamster and the isologous host and histological appearance of the tumors. A possible relationship between cytogenetic variations and biological variations in the cells is discussed.

INTRODUCTION

In our previous papers on the transplantation of the H 4-II-E rat hepatoma cells into the cheek pouches of hydrocortisone treated golden hamsters[1)2)], it was shown that significant progressive changes were observed in both phenotypes and genotypes of the cells with succeeding passages through heterologous

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hosts and tissue culture. Heterotransplantation of tissue culture cells into the cheek pouches of hydrocortisone treated hamsters resulted in the selection of cells with a fairly specific chromosomal constitution. It was also observed that the cell selection was accompanied by changes in morphology, growth rate, transplantability and a more homogeneous karyotype.

This study leaves unanswered, however, the important question as to whether the cell selection was produced by specific effects of the hydrocortisone administration and/or by alterations in the host defense mechanisms. It is generally accepted that cortisone treatment favors the transplantation of heterologous tumors by suppressing the organism's defense mechanisms, specifically antibody production and cell mediated immunity[3]. Using large doses of the hormone, even human tumors have been successfully carried in experimental animals[4)5)6)7)]. It is also well known that the use of large doses of cortisone for the purpose of depressing the immune response will inhibit the formation of new connective tissue[8)9)10)]. Transplanted tumor cells must receive a supply of stroma from the animal. This alteration of the stroma of the tumor could affect its growth.

When transplanted into A×C isologous rats, the H 4-II-E rat hepatoma cells showed markedly lower transplantability and slower growth rate in hydrocortisone treated animals than in untreated animals[11)]. Hydrocortisone administration thus seems to have two contradictory effects on the proliferation of transplanted cells in the foreign host and creates a complex interaction between the host defense mechanism and the growth of transplanted cells. These findings have indicated a need to study the cell selection mechanism using animals given another type of immunosuppressive treatment.

Since the basic work by Murphy and Taylor[12)] demonstrated that x-rays have a definite effect on the artifically induced immunity of mice to transplantable cancerous tissues, x-irradiation has been employed by many investigators for the conditioning of host animals along with cortisone administration[13)14)15)16)17)].

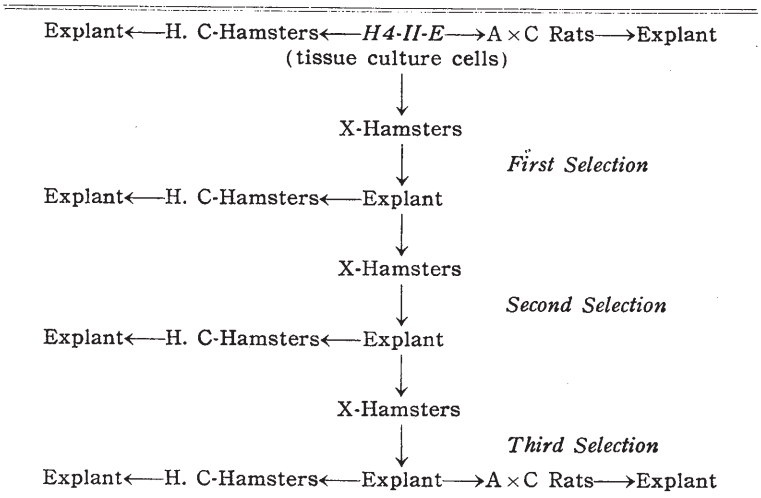
In the present study, serial heterotransplantation and re-culture was carried out three times using irradiated hamsters and the H 4-II-E rat hepatoma cell line, which exhibits a large number of differentiated functions[18)], and karyotypic and phenotypic characteristics of the cells were studied at each stage of selection both *in vivo* and *in vitro*. The primary purpose of this work has therefore been to reveal and chart any chromosomal changes in the tumor stem line during the growth of the tumor in the irradiated foreign host and tissue culture *in vitro*, and to determine possible relationships between cytogenetic variations and biological variations in the cells. It was felt that this approach might also reveal possible differences in the immunosuppressed states produced by hydrocortisone administration and x-irradiation.

MATERIALS AND METHODS

Experimental design and routine examination: The experimental design employed for this study was essentially the same as that described in the preceding reports[1)2)] (Table 1). The original cells (H 4-II-E) were implanted under the epithelium of the cheek pouches of irradiated hamsters at a concentration of 10^6 cells per pouch with a 25 gauge needle under light ether anesthesia. The animals were observed for tumor growth by everting the cheek pouch twice a week for 2 weeks following implantation. Two weeks after implantation, the animals were sacrificed and the tumors were removed. Cells from the tumors were then placed back into tissue culture (first selection). This transplant-explant experiment was repeated twice more using the irradiated animals and 4th or 5th passage generation recultured sublines (2nd and 3rd selection).

The original culture and the explants derived from tumors occurring in the irradiated animals were also implanted into the cheek pouches of hydrocortisone treated hamsters (cross transplantation: the irradiated to the hydrocortisone treated), in order to characterize the biological properties of newly established cultures, determined by the transplantability and growth characteristics in animals given another type of immunosuppressive treatment. The

TABLE 1. Experimental Design



X-Hamster: Irradiated Hamster
H. C-Hamster: Hydrocortisone Treated Hamster
Conditioning of Host:
Hydrocortisone Acetate: 3 mg, twice a week, subcutaneously, starting on the day of implantation.
X-irradiation: whole-body, 400 rads, one day before implantation.
Inoculum Size: 10^6 cells per pouch.

re-culture was attempted for the tumors in these experiments, and the changes in chromosome number were determined. This experiment was carried out to study possible differences in the immuno-suppressed states produced by hydrocortisone administration and x-irradiation.

In addition to these heterologous transplantation experiments, 10^6 cells from the original culture and re-cultured cells of the third selection were implanted subcutaneously into both thighs of untreated A×C rats. Tumor growth was evaluated twice a week for 23 days following cell implantation. Explants were established from rat tumors. This isologous transplantation experiment was designed to investigate any changes in biological and cytogenetic properties of the selected re-cultured sublines after passage through the isologous host, and to determine the degree of malignancy of the cells.

Routine examinations were carried out at each stage of selection to determine phenotypic and genotypic variations in the cells. The phenotypic characteristics studied involved transplantability into conditioned hamsters and untreated A×C rats and the histological appearance of the tumors. The cytogenetic characteristics studied included the chromosome distribution and karyotype of the explants established from tumors occurring in irradiated hamsters, hydrocortisone treated hamsters and A×C rats.

Animals and conditioning of hosts: Female golden hamsters (random bred ENG : ELA), approximately 5 weeks old and weighing between 40 and 60 gm, were employed for the heterologous tranplantations in this study. Two kinds of conditioned animals were used. The methods of conditioning of the hosts have been described elsewhere[2], and only a brief outline is given here. X-irradiation, in a dose of 400 rads total body, was given 24 hours before cell implantation. 3 mg of hydrocortisone acetate (Hydrocortone, Merk Sharp and Dohme) was given subcutaneously twice a week starting on the day of implantation. For isologous implantation, male A×C rats, ranging from 120 to 150 gm in weight, were used. Both animals were purchased from the Engle Laboratory Animals, Inc., Farmburg, Indiana.

Origin and method of culture of the rat hepatoma cell line: The H 4-II-E rat hepatoma cell line was isolated from a minimal deviation rat hepatoma, the Reuber H-35[19]. The characteristic properties of this cell line have been reported[18]. The method of culture also has been described elsewhere[2].

Histological examination: Tumors were fixed in 10% neutral formalin and stained with hematoxylin eosin and examined microscopically.

Establishment of explants from tumors and chromosome examination: Attempts were made to establish explants from all tumors. Cytogenetic characterization was carried out on the newly established explants. A detailed description of the procedure of re-culture and the method of chromosome preparation has

been previously reported[2)20)].

At the third stage of selection, an attempt was made to establish explants from two different animals in order to investigate the difference in chromosome number of explants at the same stage of selection (third selection explant # 1 and # 2).

Chromosome counts were performed on the original H 4-II-E cells and the re-cultured sublines on several occasions. As a rule, first and third passage generation cells were counted, and countings were repeated just before the cells were used for the transplantation experiments. One hundred spreads of the original culture and 50 or 100 spreads from the re-cultured sublines were examined on each occasion, and both the stem line number and the average number of chromosomes per cell were determined. Only cells with an even, round outline, that appeared to be intact and from which there was no apparent reason to assume that chromosomes had escaped, were used for chromosome counting.

Karyotyping was performed by cutting out the chromosomes and arranging them into groups according to the method used in previous papers[21)22)23)].

RESULTS

1) Serial heterologous transplant-explant experiments into the cheek pouches of conditioned hamsters and tissue culture.

Transplantability and tumor growth rate of the original H 4-II-E cells and the explants in conditioned hamsters: The growth characteristics of the H4-II-E cells in the irradiated animals at each stage of selection are represented in Table 2.

TABLE 2. Transplantability and Tumor Growth Rate of H 4-II-E and Its Re-cultured Sublines in Conditioned Hamster Cheek Pouch

Implanted Cells	Untreated Hamster	Irradiated Hamster	Hydrocortisone Treated Hamster
H 4-II-E (original culture)	0/34[A] (0.0%)	15/20 (75.0%) (8.06)[B]	16/20 (80.0%) (10.02)
Re-cultured Sublines			
First Selection Explant	0/14 (0.0%)	10/20 (50.0%) (5.22)	10/10 (100.0%) (13.46)
Second Selection Explant	0/14 (0.0%)	29/38 (76.3%) (9.10)	8/10 (80.0%) (10.39)
Third Selection Explant #1	ND	ND	17/18 (94.4%) (10.44)
#2	ND	ND	14/16 (87.5%) (13.66)

A: No. Positive/Total Inoculated, determined 2 weeks after implantation
B: Average Tumor Size, measured 2 weeks after implantation (mm)
Two explants were established from individual animal in third selection stage (third selection explant # 1 and # 2).

The original H 4-II-E cells implanted into the cheek pouches of irradiated hamsters produced tumors, which grew to maximum size approximately 2 weeks following implantation. Tumors were observed in 15 of the 20 inoculated pouches and average tumor size was 8.06 mm. At the second stage of selection, a decrease in both transplantability and average tumor size was found with the transplantability being 50% and the average tumor size 5.22 mm. At the third stage of selection, however, an accelerated growth rate was observed, and the growth behavior of the cells was almost the same as was found in the first stage of selection. No correlation was found between the rate of tumor growth and the stage of selection. The patterns of tumor growth varied from stage to stage despite the repeated transfers using animals given the same type of conditioning. Since no tumors were found in untreated animals at each stage, conditioning of the host was necessary for successful tumor growth.

The H 4-II-E cells and the explants obtained from the tumors at each stage of selection in the irradiated host were implanted into the cheek pouches of hydrocortisone treated hamsters (cross transplantation), and the results are also shown in Table 2. The rate of tumor growth of the explants at each stage of selection was equal to or higher in hydrocortisone treated animals when compared with that of the original culture. Rapid tumor growth was observed in the first selection explant and the third selection explant #2. These results stand out in sharp contrast to those in our previous papers[1)2)], in which the explants established from hydrocortisone treated animal tumors showed markedly lower transplantability than the original culture in irradiated animals.

Re-establishment of cell lines from hamster tumors: Cells derived from tumors of both the irradiated animals and the hydrocortisone treated animals could be established in tissue culture. Primary tumor cultures contained remnants of fibroblasts and blood cells from the heterologous host. The degree of contamination by host cells was higher in the explants from irradiated animal tumors than from hydrocortisone treated animals. Tumor cells grew faster than the contaminating cells, however, and the contaminating cells disappeared completely upon subculture, so that all cells appeared to be epithelial by the third or fourth passage generation. The re-cultured cells were cytologically very similar to the original H 4-II-E cells.

Histological appearance of irradiated hamster tumors: There were basically no histological differences among the tumors at each stage of selection. All tumors showed extensive hemorrhage and necrosis, and were surrounded by a wide rim of granulomatous connective tissue, consisting of fibroblasts, newly formed capillaries and inflammatory cells (polymorphonuclear leukocytes, lymehocytes and a few plasma cells). Only about 20% of the whole tumor

section was occupied by typical tumor cells. Occasionally foreign body giant cells were present in the granulomatous tissue. The tumor cells were polygonal or oval with abundant basophilic cytoplasm, large round nuclei and big nucleoli. The histological appearance of a tumor at the first stage of selection is illustrated in Figure 1.

It is of interest to note that these histological findings are markedly different from those of hydrocortisone treated animal tumors, shown in our previous papers[1)2)]. The tumors of the hydrocortisone series showed a more monotonous and well differentiated histological appearance with each succeeding passage. The tumors of the present series were characterized by more hemorrhage and necrosis with marked granulomatous reaction and cell variation in size and shape, without disclosing any progressive changes with each succeeding transfer through the heterologous host and tissue culture.

Chromosome distribution of the H4-II-E cells and the explants derived from tumors occurring in irradiated hamsters: It was important to determine the exact chromosome makeup of the original cell line before starting this serial transplant-explant experiment. One hundred cells were counted (July, 1970), and it was found that the cell population of this culture consisted of a spectrum of hypotriploid cells which had a tendency to be bimodal (the top of Table 3). The average chromosome number per cell was 50.25, and showed a wide distribution as is frequently found in established permanent cell line.

The results of chromosome counts of the explant at each stage of selection are also presented in Table 3. Chromosome counts were carried out three times for the explant at the first stage of selection. The modal chromosome number was shifted upwards from 51 to 53, and the scatter of cells around the stem line appeared to be less for the explant than for the original culture.

At the second and third stage of selection, almost the same distribution of chromosomes was observed as that found in the first stage. The differences between these explants were found in the modal chromosome number. The explant from the second stage of selection had a modal chromosome number of 54. The two explants from the third stage of selection each showed different modal chromosome number (53 and 54).

These results indicate that the modal chromosome number of each explant varies from stage to stage, even among the explants from different animals at the same stage of selection. They did not show any progressive changes in chromosome number despite the fact that *in vivo* and *in vitro* selection pressures were given repeatedly. As already mentioned, the contamination of the primary explants by host cells was observed during the early stage of the culture. It is of some interest that the cultures containing host cells tended to reveal a bimodal distribution with approximately equal numbers of cells having either 53 or 54 chromosomes, also very few cells showed a

TABLE 3. Chromosome Distribution of H 4-II-E and Its Explants Derived from Tumors in Irradiated Hamsters

Culture	Passage Number *in vitro*	Chromosome Numbers														Total No. of Cells Counted	Average Chromosome Number	Remarks
		≦45	46	47	48	49	50	51	52	53	54	55	56	60	≧61			
H 4-II-E (July, 70)		5	4	5	10	11	12	16*	8	12	10	4	1	1	1	100	50.25	
First Selection Explant	1	4.8						4.8	2.4	42.9*	45.2*					42	52.74	mixed fibroblasts
	3					8	2	4	4	42*	36	4				50	52.94	
	23	2				2		6	12	46*	28		4			50	52.86	
Second Selection Explant	3					2	4	8	4	22	48*	10	2			50	53.34	
	13				4	4			8	28	48*	8				50	53.20	
Third Selection Explant #1	3	1	1	3	2	1		5	7	36*	37*	4	2	1		100	52.95	mixed fibroblasts
	8	2				2	2		8	24	48*	14				50	53.28	
Third Selection Explant #2	3	4		2	2	2		4	8	32*	32*	14				50	52.70	mixed fibroblasts
	5					2		16	6	42*	26	8				50	52.96	

Results are expressed as per cent of cells with the indicated number of chromosomes.

Two explants were established from individual animal in third selection stage (third selection explant #1 and #2).

* modal chromosome numder

chromosome number corresponding to that of the diploid number for the hamster (=44). Though chromosomal changes may occur spontaneously, the present results clearly indicate that the repeated serial transfer of the H 4-II-E cells through irradiated hamsters and tissue culture was accompanied by a change in the distribution of the chromosome number, the mode having increased to 53 or 54 and the average chromosome number showing only a narrow variation around 53. This increase in stem line number and narrowing of the scatter of the chromosome number also suggest that there is some selection going on in the irradiated animals for cells with a particular genetic makeup.

Changes in chromosome number of the original H 4-II-E cells and the explants after transfer through hydrocortisone treated hamsters: In order to characterize the biological and cytogenetic properties of the re-cultured sublines, which had different chromosomal patterns from that of the original culture, all of the explants were implanted into the cheek pouches of hydrocortisone treated hamsters. H 4-II-E cells were also injected into hydrocortisone treated animals as a control experiment. Re-culture was carried out for every experimental group, and the chromosome numbers were determined using the primary and third passage generation cultures. Results of these counts both before and after implantation are given in Table 4.

As previously reported[1)2)], marked changes in chromosome number could be found in the experimental group receiving the original H 4-II-E cells. The modal chromosome number was shifted upwards from 51 to 53 and 54 with a very narrow range of variation. No significant changes in either modal chromosome number or average chromosome number per cell were observed in the explants from the irradiated animals except in the second selection explant.

These results demonstrate that the cells passed through irradiated animals persist unchanged in chromosome number even when they are injected into hydrocortisone treated hamsters, showing that there are marked differences in genotype between the original H 4-II-E culture and the re-cultured sublines.

Karyotype analysis of the H 4-II-E cells and four explants derived from tumors growing in the cheek pouch of irradiated hamsters: Classification of chromosomes was carried out according to the criteria described by Hungerford *et al.*[21)22)23)]. Karyotyping was carried out on the metaphase plates of the stem line chromosome number at each stage of selection. Well-spread metaphase plates were selected from the slides used to examine the chromosome distribution. Early cultures containing host cells were not used for karyotype analysis. It is not always easy to identify the individual chromosomes assigned to groups 1-3, 4-10, X.Y., 11-13 and 14-20 and, furthermore, it is very difficult to arrange them into homologous pairs because the tumor chromosomes

TABLE 4. Chromosomal Changes of H 4-II-E and Its Explants Derived from Irradiated Hamster Tumors After Implantation Into Hydrocortisone Treated Hamsters

Implanted Cells	Passage Number *in vitro*	Chromosome Number ≤40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	60	≥61	Total No. of Cells Counted	Average Chromosome Number	Remarks
	—	1		1	1	2		4	5	10	11	12	16*	8	12	10	4	1		1	1	100	50.25	B.T.
H 4-II-E	1								4.7				4.7	14	25.6	46.5			4.7			43	53.14	A.T. (mixed host cells)
	3	2		2					2	4	10		2	4	38*	30	4		2			50	52.02	A.T.
	23			2							2		6	12	46*	28		4				50	52.86	B.T.
First Selection Explant	1							2			4	8	8	16	38*	18	2	4				50	52.48	A.T. (mixed host cells)
	3					2	2			2	4		12	16	34*	20	4	4				50	52.40	A.T.
	13									4	4			8	28	48*	8					50	53.20	B.T.
Second Selection Explant	1							2			6		12	8	24	38*	2	2	4		2	50	53.16	A.T. (mixed host cells)
	3	2								2	2	2	8	6	40*	34	4					50	52.70	A.T.
	8	2									2	2		8	24	48*	14					50	53.28	B.T.
#1	1	2							2	2	6		16	4	28*	28*	10		4			50	52.48	A.T. (mixed host cells)
Third Selection Explant	3								2		2		2	4	32	44*	8	2	4			50	53.54	A.T.
	5										2		16	6	42*	26	8					50	52.96	B.T.
#2	1			2		4	4			2	2		18	6	40*	18	4					50	51.76	A.T. (mixed host cells)
	3					2							8	6	42*	32	8	2				50	53.16	A.T.

Results are expressed as per cent of cells with the indicated number of chromosomes.
Primary flasks mixed host cells, however, they were much less than those in explants derived from irradiated hamster tumors.
*: modal chromosome number B.T.: Before transplantation A.T.: After transplantation

TABLE 5. Karyotype Variation in the H 4-II-E and Its Explants Derived from Irradiated Hamster Tumor

Culture	Total Number	1-3	4-10, X.Y.	Group 11-13	14-20	Marker Chromosome
H 4-II-E	44	6	21	7	10	0
	49	7	17	9	15	1
	50	6	19	7	17	1
	50	6	20	8	15	1
	50	6	21	8	14	1
	51	6	22	6	16	1
	51	5	19	8	18	1
	51	7	21	7	15	1
	51	6	18	9	18	1
	53	6	19	9	18	1
	53	6	19	10	17	1
	53	6	19	9	18	1
	54	6	23	7	17	1
	54	6	21	8	18	1
	54	6	21	8	18	1
	54	6	20	9	18	1
First Selection Explant	53	6	18	11	17	1
	53	6	18	11	17	1
	53	6	18	10	18	1
	53	6	18	10	18	1
	53	6	19	10	17	1
	53	6	19	10	17	1
	53	6	17	12	17	1
	53	6	20	10	16	1
Second Selection Explant	54	6	19	10	18	1
	54	6	19	10	18	1
	54	6	19	10	18	1
	54	6	19	10	18	1
	54	6	19	10	18	1
	54	6	19	10	18	1
	54	6	20	10	17	1
Third Selection Explant #1	54	6	19	10	18	1
	54	6	19	10	18	1
	54	6	19	10	18	1
	54	6	19	10	18	1
	54	6	19	10	18	1
	54	6	18	11	18	1
	54	6	18	11	18	1
Third Selection Explant #2	53	6	18	10	18	1
	53	6	18	10	18	1
	53	6	17	11	18	1
	53	6	17	10	19	1
	53	6	19	10	17	1

appear to have undergone marked changes in morphology during the development of the tumor. Results of the karyotype analysis are tabulated in Table 5.

H 4-II-E (mode of 51): Nine metaphase plates were chosen to make the detailed karyotypes. Moreover, 7 metaphase plates with 53 and 54 chromosomes were used to make karyotypes in order to compare with those of the re-cultured sublines. The number of chromosomes in each group varied from cell to cell, even among the cells with identical chromosome numbers, except

group 1-3, which showed a relatively stable pattern. One marker chromosome that is never found in the normal rat karyotype was observed in 15 of 16 cells. This marker chromosome is the largest submetacentric observed in the material and was clearly distinguishable from other chromosomes.

First selection explant (mode of 53): The stem line karyotypes of the culture varied considerably and deviated from those of the original cells with 53 chromosomes. Five different karyotype patterns were found in 8 metaphase plates analysed. The large marker chromosome found in the original culture was seen in all cells examined.

Second selection explant (mode of 54): Karyotyping was carried out on 7 metaphase plates. It is of interest that 6 of 7 plates showed the same chromosome pattern. Deviation of the karyotype from diploid are as follow: group 4-10, X.Y. (+3); group 11-13 (+4); group 14-20 (+4) and 1 marker chromosome. Although a homogeneous karyotype was observed in this culture, it was markedly different from those of the original H 4-II-E cells with 54 chromosomes.

Third selection explant # 1 (mode of 54): Minor deviations in the number in each group were observed. Only two different karyotypes were found in the stem line karyotypes of this culture. The same chromosome arrangement was observed in 5 of 7 plates examined and was also exactly the same as the major karyotype of the second selection explant. It appears that the second selection explant and the third selection explant # 1 have almost the same chromosomal constitutions, as judged by modal number and karyotype.

Third selection explant # 2 (mode of 53): Five metaphase plates were selected to make the karyotypes and four different patterns were observed in the stem line karyotype. Two of 5 cells had the same karyotype and the 3 other cells each had different karyotypes.

In summary, it is of special interest to note that the re-cultured sublines with 54 modal chromosome number had a more homogeneous karyotype than cells with 53 chromosomes, showing exactly the same karyotype arrangement as that of the explant derived from hydrocortisone treated animal tumors[1)2)]. It is also apparent that the stem line karyotypes of each explant are not similar to that of the original H 4-II-E culture. No morphologic differences in the chromosomes of the original cells and these explants could be detected.

2) Isologous implantation of the original H 4-II-E cells and the third selection explants into A × C rats.

There no longer seems to be any doubt that isologous transplantation is the best experimental tool to determine the degree of malignancy of cells. The third selection explant # 1 and # 2 were chosen for isologous transplantation experiment. The former was characterized by a modal chromosome number of 54 and a homogeneous karyotype population and the latter by a modal chromosome number of 53 and a heterogeneous karyotype population.

The original culture was also implanted into A × C rats as a control experiment.

Transplantability and tumor growth rate in isologous hosts: The average tumor size and transplantability were determined on the 17th and 23rd day after implantation, respectively. The results are shown in Table 6. The third selection explant # 1 showed lower transplantability and slower growth rate than the other two implanted cultures. The highest transplantability and the fastest tumor growth were observed in the third selection explant # 2. It appears from these results that the third selection explant # 1 is less malignant when compared with the other two cultures, as judged by transplantability and tumor growth rate in the isologous host (A × C rat).

TABLE 6. Transplantability and Tumor Growth Rate of H 4-II-E and Third Selection Explants in A × C Rats

Implanted Cells	Transplantability–(23rd day)	Average Tumor Size (mm) (17th day)
H 4-II-E	13/16 (81.3%)	10.89
Third Selection Explant # 1	8/16 (50.0%)	5.12
Third Selection Explant # 2	16/16 (100.0%)	13.21

A: No. Positive/Total Inoculated
Animal body weight: 130–150 gm Inoculum size: 10^6 cells
Average tumor size and transplantability were determined on the 17th day and 23rd day after implantation, respectively.

Histological appearance of rat tumors:

H 4-II-E (Figure 2): The tumors showed moderate central hemorrhage and necrosis with fibrous reaction. The tumor cells revealed moderate variation in size and shape. Bizarre mitotic figures were prominent. Tumor giant cells were absent. Balloon degeneration of tumor cells was found in scattered areas. The individual tumor cells were polygonal or oval with eosinophilic to basophilic granular cytoplasm. Bile stasis was absent. The tumor cells lined vascular sinuses several to many cells thick.

Third selection explant # 1 (Figure 3): The individual tumor cells were smaller with dense basophilic cytoplasm, and were more compact with a monotonous picture. Tumor giant cells, pseudotubule and pseudoacinus formation and balloon degeneration were absent.

Third selection explant # 2 (Figure 4): The sections of the tumors were similar to those of the original culture rather than to those of the third selection explant # 1. The tumor showed mild to moderate hemorrhage and necrosis with occasional fibrous reaction. The individual tumor cells were polygonal with basophilic granular cytoplasm. Bizarre mitotic figures were prominent. Pseudotubule and pseudoacinus formation and balloon degeneration were found.

Histological findings of these three groups are summarized in Table 7. There were no definite differences among the tumors produced by the H 4-II-E and the third selection explant #2. The tumors produced by the third selection explant #1 were apparently different from the others; the individual tumor cells were smaller, more compact with dense basophilic cytoplasm. The tumors of this group are thought to be less malignant than the others.

TABLE 7. Histological Findings of Rat Tumors

Transplanted Cells	H 4-II-E	Third Selection Explant #1	Third Selection Explant #2
Hemorrhage and Necrosis	++	+	++~+++
Fibrous Reaction	+~++	−~+	++
Cell Arrangement	Essentially same: the tumor cells line vascular sinuses several to many cells thick		
Tumor Cells	eosinophilic to basophilic, granular	smaller, dense basophilic, less much cytoplasma	basophilic, granular
Bizarre Mitotic Figures	++	−~+	++
Pseudotubule and Pseudoacinus Formation	++~+++	−~+	++~+++
Balloom Degeneration	++	−~+	++

Giant cells and bile stasis are absent in all tumors.

Chromosome distribution of the explants derived from rat tumors: Before attempting the isologous implantation, chromosome examination was carried out on the original H 4-II-E cells and the two explants (July, 1971). Re-culture was undertaken on the 17th day after implantation to investigate the changes in chromosome number after transfer through the isologous host. Contamination of host cells was found in primary culture flasks. The contamination by host cells was, however, much less than in the explants derived from the heterologous animal tumors and disappeared upon subculture.

Chromosomes were counted on two occasions after establishing the new cultures from the rat tumors. The results of these counts are illustrated in Table 8. Certain chromosomal changes were detected in the original H 4-II-E cells between the result on July, 1970 and that on July, 1971. The modal chromosome number was shifted upwards from 51 to 53 and 54, but a wide distribution and a tendency to be bimodal were still present. In the experimental groups of the original culture and the third selection explant #2, minimal changes were observed in both stem line number and average chromosome number per cell after passage through the isologous host. The third selection explant #1 showed less stable patterns in chromosome number

TABLE 8. Chromosomal Changes of H 4-II-E and Third Selection Explants After Implantation Into A×C Rats

Implanted Cells	Passage Number *in vitro*	Chromosome Number																		Total No. of Cells Counted	Average Chromosome Number	Remarks	
		40	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58				
H 4-II-E	July, 71		1		4				4	3	2	13	7	29*	27*	5	3	1	1	100	52.37	B.T.	
	1								2	2		6	2	20	50*	12	6			50	53.60	A.T.	(mixed host cells)
	3				2	2			2		2	18	6	28*	26*	10	2	2		50	52.68	A.T.	
Third Selection Explant #1	8	2								2	2		8	24	48*	14				50	53.28	B.T.	
	1				2	4	2			6	4	10	4	16	40*	8	4			50	52.44	A.T.	(mixed host cells)
	5			2			2	2	2		2	8	26*	24*	26*	6				50	52.30	A.T.	
Third Selection Explant #2	5									2		16	6	42*	26	8				50	52.96	B.T.	
	1								2		2	20	6	48*	14	6	2			50	52.70	A.T.	(mixed host cells)
	3					2		2		2		4	20	56*	8	2			2	50	52.68	A.T.	

Results are expressed as per cent of cells with the indicated number of chromosomes.
Primary flasks mixed host cells, however, they were much less than those in explants derived from heterologous animal tumors.
*: modal chromosome number B.T.: Before transplantation A.T.: After transplantation

compared with the other two cultures. It may generally be concluded from these results that fewer changes in chromosome number occur in the isologous host than in the heterologous host, even if immunosuppressive procedures are given to the heterologous animals.

DISCUSSION

Much information regarding chromosomal changes in tumor cells after heterologous transplantation have been reported by various workers. Some investigators found that such tumors, even if maintained by serial passage in heterologous hosts, retain their cytogenetic characteristics[24)25)26)]. Conversely, other investigators have indicated difinite changes in the chromosomal constitution of the cell population of transplanted tumor cells after growth in different species or strain[27)28)29)30)31)32)33)]. These observations were ascribed to a selection of the cell best able to adapt themselves and grow in the new environment[27)30)31)34)], and may also be accompanied by a change in transplantability and tumor growth rate[34)35)].

The present experiments show that transplantation of the H 4-II-E rat hepatoma cells into the cheek pouches of irradiated hamster resulted in an increase in modal chromosome number with a narrow range of distribution. The explants in the first and second stage of selection were characterized by 53 and 54 chromosomes, respectively. The two explants derived from individual animal tumors in the third stage of selection showed different chromosomal patterns from each other (modes of 53 and 54). The explants with 54 chromosomes revealed a much more homogeneous karyotype population than did those with 53 chromosomes. The patterns of chromosomal change of the cells passed through irradiated animals thus varied from stage to stage, even among explants from different animals at the same stage of selection, without disclosing any progressive change in chromosome number with each succeeding transfer as seen previously in hydrocortisone treated hamsters[1)2)]. The changes appeared to be so random that it is difficult to decide whether these changes should be attributed to mutation or selection or to a combination of both. The possibility also exists that the capacity of the original cells to undergo serial heterotransplantation may be a function of a viral oncogenesis, in which case the karyotype of the transplanted tumor cells would be expected to resemble the karyotype of the host. Moreover, the appearance of tumors with recipient host genotype following the transplantation of malignant tissues from a donor of different strain or species has been demonstrated by many workers[35)36)37)38)39)].

However, a mutation involving the whole-chromosome set must be considered extremely unusual, the sudden change might have been due to contamination with a spontaneous tumor, or to the development of a virus-induced

tumor[31]. The modal chromosome number of each explant is 53 or 54, and the tendency to shift to 53 and 54 chromosome number was also observed in serial transplantation using the H 4-II-E cells and hydrocortisone treated hamsters, in which progressive chromosomal changes were found with each succeeding passage and, in the final stage, more than 90% of the cells had 53 or 54 chromosomes associated with very homogeneous karyotypes[1)2)]. No morphologic differences in the chromosomes between the original H 4-II-E cells and each explant could be observed. Furthermore, electron microscopic examination of both tumors and explants, to be reported in detail later, have shown no morphologic evidence for the presence of virus or mycoplasma. It seems therefore difficult to imagine that the chromosomal changes are due to mutation or intraspecies hybridization or viral oncogenesis. It is more likely that environmental changes are capable of inducing chromosomal changes in a tumor and these changes might possibly be due to a selection of cells already present in the original tumors.

Since little is known about the cellular interaction between the hamster cells and this particular cell line, the explanation for chromosomal changes in the process of going from the mixed culture (early stage of establishment of the explant) to the epithelial culture (Table 3 and 4) is not evident. The presence of so many host cells may exert some influence upon the chromosomal constitution of the tumor cells. Further studies into this problem are necessary. One possible explanation is an alteration of the tissue culture environment. Very few cells in mixed cultures showed the chromosome number corresponding to that of the diploid number for the hamster. This could be interpreted as due to the resistance of the hamster cells to the stathmokinetic action of colchicine and its derivatives[40)41)42)43)].

The question also arises as to why the differences in chromosomal pattern were observed in the explants of each stage and even explants from the same stage, and why the explants did not show any progressive change in chromosomal pattern in spite of repeated transfers through animals conditioned by the same treatment, assuming that the chromosomal changes are due to cell selections.

In order to discuss this problem, we must consider the immunosuppressed state produced by X-irradiation. Maximal depression of the immune response is produced by irradiating the animal a few hours to a few days before antigenic stimulation[44)]. Human cancer cells show a higher percentage of "takes" and proliferate considerably longer in cortisone treated and cortisone treated, irradiated animals than in hosts which have received irradiation treatment alone[45)46)]. When the original H 4-II-E cells were transplanted into the cheek pouches of hamsters 7 and 14 days following irradiation treatment (400 rads, whole-body), no tumors were observed[47)]. This result would indicate that irradiated animals may recover from the immunosuppressed state within one week following the treatment. The suppression of host resistance by

irradiation thus appears to be very temporary. It seems likely to assume that proliferating tumor cells in the cheek pouches of irradiated animals would be challenged by an immune response from about one week following implantation, and, as the degree of recovery from the immunosuppressed state may vary from animal to animal, some tumors may be subjected to greater immuno-selection than others. Though our limited data do not permit much speculation at this time, if this assumption is correct, it would be easy to explain the differences in the chromosomal patterns of each explant and in the growth behavior of the tumor cells at each stage. This assumption is also supported by the histological appearance of the tumors with all tumors having marked hemorrhage and necrosis, surrounded by a wide rim of granulomatous connective tissue, with no definite differences among the tumors in each stage.

With the data so far available we can not define the exact role of hydrocortisone in the selection of cells because the exact mechanisms of the effects of hydrocortisone and X-irradiation are as yet unknown although both result in immuno-suppression probably by suppressing cell mediated immunity. Hydrocortisone does, however, have other metabolic effects which might also play a role in cell selection. The differences in the pattern of the selection of cells, the tumor growth behavior at each stage and the histological appearance of tumors suggest that a marked difference exists between the immunosuppressed states induced by hydrocortisone administration[1)2)] and X-irradiation. For more conclusive information on this point, further study will be required using untreated and hydrocortisone treated isologous animals.

Apart from the chromosomal changes which occurred by serial heterotransplantation of the H 4-II-E cells through the irradiated animals, one of the most interesting results of this study was the difference in growth behavior and histological findings in the isologous transplantation experiments. Although a considerable number of publications concerning the cytogenetic characteristics of tumor cells are available, little is known about the relationship of variation in number and type of specific chromosomes with variation in biological characteristics of cancer cells. Of specific interest here are the cytogenetic characteristics of neoplastic cells. Malignant cell populations are highly heterogeneous chromosomal mosaics[48)49)]; the clonal derivatives established by single cell transplantation have different modes of chromosome number[50)51)52)]; the chromosome number of transplantable tumors shows wider distribution[53)54)55)] than normal adult or embryonic materials[55)56)]. It is also generally accepted that a significant relationship exists between chromosome constitution and histocompatibility in a variety of transplantable tumors[57)58)59)]. The occurrence of increasing virulance of neoplastic cells is regularly accompanied by a decrease in host specificity, and by changes in chromosome number[58)]. In every tumor the major part of the cells have a certain chromosome number called stem line number, and this number persists unchanged for a long time

on isologous transplantation and these cells may thus be regarded as reproducing the tumors[60)61)].

Results from the isologous trausplantation experiments are of interest in regard to this point. It is apparent that isologous transplantation is the best way to determine the degree of malignancy of cells. The original H 4-II-E cells and two recultured sublines (third selection explant #1 and #2), which have markedly different chromosomal constitutions, were transplanted into A×C rats subcutaneously. The third selection explant #1, which has a mode of 54 with a homogeneous stem line karyotype, showed markedly lower transplantability and slower growth rate than did the third selection explant #2, which is characterized by a mode of 53 and heterogeneous stem line karyotypes. The original cells have a bimodal chromosome distribution (53 and 54) with a wider range, and its growth rate in the rats was between those of the third selection explant #1 and #2. It is clear from these data that the third selection explant #1 is less malignant than the original cells and the third selection explant #2. This finding is also supported by the histological appearance of the rat tumors. The tumors produced by the third selection explant #1 appear less malignant than tumors produced by the original cells and the third selection explant #2 (Table 7). The results described above strongly support our assumption in earlier works[1)2)] that the uniformity of karyotype and the width of chromosome distribution as well as modal chromosome number and ploidy and morphology of chromosome may control the biological properties of cells to some degree.

In the experiment in which cells were injected into A×C rate (isologous transplantation) fewer chromosomal changes were observed. These results support the concept that environmental changes can result in a change in the chromosomal makeup of the cancer cells[30)31)], since environmental changes are more pronounced on heterologous than on isologous transplantation, although, as Haldane has pointed out[62)], differences in environment occur even on isologous transplantation.

The fewer chromosomal changes seen on the cross transplantation experiment may also be explained by adaptation of the tumors to immunogenetic environment of the new host[34)]. It is reasonable to assume that the cells derived from tumors growing in irradiated hosts have a greater capability to grow in the hydrocortisone treated animals than do the original cells because they have already been subjected to a foreign environment, though there are some indications suggesting that there may be marked differences between the immunosuppressed states induced by these two modalities.

Two main points stand out as a result of our studies: (a) transplantation of the H 4-II-E rat hepatoma cells into the cheek pouch of irradiated hamsters results in the selection of cells with a specific chromosomal constitution, while the pattern of selection of cells is markedly different from that in hydro-

cortisone treated animals. (b) there is apparently a relationship of the karyotypes and number of specific chromosomes with variations in the biological properties of the cells.

REFERENCES

1) Muragishi, H., Lovig, C. A., Corpening, B. and Bottomley, R. H., The Relationship of Karyotype to Phenotype in Variants of the H 4-II-E Rat Hepatoma Cell Line, *In Vitro*, **6**, 391, 1971 (Abstr.).
2) Muragishi, H. and Bottomley, R. H., Effect of Heterologous Transplantation of H 4-II-E Rat Hepatoma Cells into the Cheek Pouch of Hydrocortisone Treated Hamsters, *Nagoya J. Med. Sci.*, **36**, 49, 1973.
3) Bradley, J. and Elson, C. J., Suppression of the Immune Response, *J. Med. Genetics*, 8, 321, 1971.
4) Toolen, H. W., Transplantable Human Neoplasms Maintained in Cortisone-treated Laboratory Animals; H. S. #1; H. Ep. #1; H. Ep. #2; H. Ep. #3 and H. Emb. Rh. #1, *Cancer Res.*, **14**, 660, 1954.
5) Patterson, W. B., Chute, R. N. and Sommers, S. C., Transplantation of Human Tumors into Cortisone-treated Hamsters, *Cancer Res.*, **14**, 656, 1954.
6) Handler, A. H., Davis, S. and Sommers, S. C., Heterotransplantation Experiments with Human Cancers, *Cancer Res.*, **15**, 32, 1955.
7) Patterson, W. B., Transplantation of Human Cancers to Hamster Cheek Pouches, *Cancer Res.*, **28**, 1637, 1968.
8) Ragan, C., Howes, E. L., Plotz, C. M., Meyer, K. and Blunt, J. W., Effect of Cortisone on Production of Granulation Tissue in the Rabbit, *Proc. Soc. Exp. Biol., N. Y.*, **72**, 718, 1949.
9) Baker, B. L. and Whitaker, W. L., Interference with Wound Healing by the Local Action of Adrenalcortical Steroids, *Endocrinology*, **46**, 544, 1950.
10) Spain, D. M., Molomut, N. and Haber, A., Biological Studies on Cortisone in Mice, *Science*, **112**, 335, 1956.
11) Muragishi, H. and Bottomley, R. H., Unpublished Observation.
12) Murphy, J. B. and Taylor, H. D., The Lymphocyte in Natural and Induced Resistance to Transplanted Cancer. III. The Effect of X-Rays on Artificially Induced Immunity, *J. Exper. Med.*, **28**, 1, 1918.
13) Toolan, H. W., Successful Subcutaneous Growth and Transplantation of Human Tumors in X-irradiated Laboratory Animals, *Proc. Soc. Exper. Biol. and Med.*, **77**, 572, 1951.
14) Toolan, H. W., Proliferation and Vascularization of Adult Human Epithelium in Subcutaneous Tissues of X-irradiated Heterologous Hosts, *Proc. Soc. Exper. Biol. and Med.*, **78**, 540, 1951.
15) Toolan, H. W., Growth of Human Tumors in the Subcutaneous Tissue of X-irradiated Laboratory Animals; Their Practical Use for Experimental Purposes, *Cancer Res.*, **12**, 302, 1952.
16) Sommers, S. C., Chute, R. N. and Warren, S., Heterotransplantation of Human Cancer. I. Irradiated Rats, Cancer Res, *Cancer Res.*, **12**, 909, 1952.
17) Adams, R. A., Farber, S., Foley, G. E., Uzman, B. G., Lazarus, H. and Watrouse, P., Heterotransplantation of Human Leukemic Cells and Cell Cultures in the Lathally X-irradiated Syrian Hamster, *Cancer Res.*, **26**, 2190, 1966.
18) Pitot, H. C., Peraino, C., Morse, P. A. Jr. and Potter, V. R., Hepatomas in Tissue Culture Compared with Adapting Liver *in vitro*, *Natl. Cancer Inst. Monogr.*, **12**, 229, 1964.

19) Reuber, M. D., A Transplantable Bile-secreting Hepatocellular Carcinoma in the Rat, *J. Natl. Cancer Inst.*, **26**, 891, 1961.
20) Bottomley, R. H., Trainer, A. L. and Griffin, M. J., Enzymatic and Chromosomal Characterization of HeLa Variants, *J. Cell Biol.*, **41**, 807, 1969.
21) Hungerford, D. A. and Nowell, P. C., Sex Chromosome Polymorphism and the Normal Karyotype in Three Strains of the Laboratory Rat, *J. Morphol.*, **113**, 275, 1961.
22) Nowell, P. C., Ferry, S. and Hungerford, D. A., Chromosomes of Primary Granulocytic Leukemia (Chloroleukemia) in the Rat, *J. Natl. Cancer Inst.*, **30**, 687, 1963.
23) Nowell, P. C., Morris, H. P. and Potter, V. R., Chromosomes of "Minimal Deviation" Hepatomas and Some Other Transplantable Rat Tumors, *Cancer Res.*, **27**, 1563, 1967.
24) Sachs, L., and Gallily, R., The Chromosomes and Transplantability of Tumors. I. Fundamental Chromosome Numbers and Strain Specificity in Ascites Tumors, *J. Natl. Cancer Inst.*, **15**, 1267, 1955.
25) Stroud, A. N., Brues, A. M., Chatterley, D. H. and Sommers, M., Serial Transplantation of Krebs-2 and Ehrlich Ascites Tumors to Rats, *Cancer Res.*, **17**, 1102, 1957.
26) Miles, C. P., Chromosomes of Some Heterotransplanted Human Tumors. I. H. Emb. Rh. #1, H. S. #1 and ME 1, *J. Natl. Cancer Inst.*, **34**, 103, 1965.
27) Ising, U., Chromosome Studies in Ehrlich Mouse Ascites Cancer after Heterologous Transplantation through Hamsters, *Brit. J. Cancer*, **9**, 592, 1955.
28) Levan, A., Chromosomes in Cancer Tissue, *Ann. N. Y. Acad. Sci.*, **63**, 774, 1956.
29) Hauschka, T. S., Kvedar, B. Jr., Grinnell, S. T. and Amos, D. B., Immunoselection of Polyploids from Predominantly Deploid Cell Populations, *Ann. N. Y. Acad. Sci.*, **63**, 683, 1956.
30) Ising, U., Chromosomal Changes in Ehrlich's Mouse Ascites Cancer on Repeated Transfer through Hamsters and Rats, *Acta Path. Microbiol. Scand.*, **40**, 315, 1957.
31) Ising, U., Effect of Heterologous Transplantation on Chromosomes of Ascites Tumors. A Contribution to Our Knowledge of Environmental Influence on Tumor Cells, *Acta Path. Microbiol. Scand. Suppl.*, **127**, 1, 1958.
32) Galton, M., Goldman, P. B. and Holt, S. F., Karyotypic and Morphologic Characterization of a Serially Transplanted Human Choriocarcinoma, *J. Natl. Cancer Inst.*, **31**, 1019, 1963.
33) Krishan, A., Raychaudhuri, R. and Flowers, A., Karyotype Studies on Human Leukemic Lymphoblasts *in vitro* and as Serial Transplants in Neonatal Syrian Hamsters, *J. Natl. Cancer Inst.*, **43**, 1203, 1969.
34) Ahlstrom, C. G. and Ising, U., Alteration in Growth of Ehrlich Mouse Ascites Carcinoma on Repeated Transfer through Hamsters, *Brit. J. Cancer*, **9**, 582, 1955.
35) Suciu-Foca, N., Dumitrescu, V., Lazar, C. and Nachtigal, M., Host and Tumor Modifications Associated with Serial Heterotransplantation of Tumors through Immnologically Tolerant Animals, *Cancer Res.*, **30**, 1681, 1970.
36) Iversen, H. G., Transplantation from Man to Mouse of Exudates Containing Tumor Cells, *Brit. J. Cancer*, **12**, 210, 1958.
37) de Pasqualini, C. D., Pavlovsky, A., Holmberg, E. A. D. and Rabasa, S. L., Development of Murine Leukemia after Inoculation of Human Lymphomas, *Cancer Res.*, **28**, 788, 1968.
38) Goldenberg, D. M. and Gotz, H., On the "Human" Nature of Highly Malignant Heterotransplantable Tumors of Human Origin, *European J. Cancer*, **4**, 547, 1968.
39) Green, H. S. N. and Harvey, K. E., The development of Sarcomas from Transplants of the Hyperplastic Stromal Endothelium of Glioblastoma Mutiform, *Am. J. Pathol.*, **53**, 483, 1968.
40) Orsini, M. W. and Pansky, B., The Natural Resistance of the Golden Hamster to Colchicine, *Science*, **115**, 88, 1952.

41) Turbyfill, C. L. and Sonderwall, A. L., Sensitivity of Hamster to Colchicine, *Science*, **126**, 749, 1957.
42) Cardinali, G., Cardinali, G., Handler, A. H. and Agritoglio, M. F., Comparative Effects of Colchicine and Vincaleukoblastine on Bone Marrow Mitotic Activity in Syrian Hamster, *Proc. Soc. Exper. Biol. and Med.*, **107**, 891, 1961.
43) Goldenberg, D. M., Stathmokinetic Effect of Colcemide on a Presumptive Human-Hamster Hybrid Tumor, GW-478, *Exptl. Mol. Pathol.*, **14**, 134, 1971.
44) Berenbaum, M. C., The Effect of Cytotoxic Agents on the Production of Antibody to TAB Vaccine in the Mouse, *Biochemical Pharmacology*, **11**, 29, 1962.
45) Toolan, H. W., Growth of Human Tumors in Cortisone-treated Laboratory Animals: The Possibility of Obtaining Permanently Transplantable Human Tumors, *Cancer Res.*, **13**, 389, 1953.
46) Toolan, H. W., Conditioning of the Host, *J. Natl. Cancer Inst.*, **14**, 745, 1953.
47) Muragishi, H. and Bottomley, R. H., Unpublished Observation.
48) Hauschka, T. S., Immunologic Aspects of Cancer, *Cancer, Res.*, **12**, 615, 1952.
49) Hauschka, T. S., Methods of Conditioning the Graft in Tumor Transplantation, *J. Natl. Cancer Inst.*, **14**, 723, 1953.
50) Hauschka, T. S., Cell Population Studies in Mouse Ascites Tumors, Trans. *New York Acad. Sci., Ser. II*, **16**, 64, 1953.
51) Sato, H., On the Chromosomes of Yoshida Sarcoma. Studies with Tumor Cells Proliferated in the Peritoneal Cavity of the Rat Transplanted with a Single Cell, *Gann*, **43**, 1, 1953.
52) Hauschka, T. S. and Levan, A., Cytologic and Functional Characterization of Single Cell Clones Isolated from the Krebs-2 and Ehrlich Ascites Tumors, *J. Natl. Cancer Inst.*, **21**, 77, 1958.
53) Makino, S., A Cytological Study of the Yoshida Sarcoma, an Ascites Tumor of White Rats, *Chromosoma*, **4**, 649, 1952.
54) Levan, A. and Hauschka, T. S., Chromosome Number of Three Mouse Ascites Tumours, *Hereditas*, **38**, 251, 1952.
55) Levan, A. and Hsuschka, T. S., Endomitotic Reduplication Mechanisms in Ascites Tumors of the Mouse, *J. Nutl. Cancer Inst.*, **14**, 1, 1953.
56) Tanaka, T., A Study of the Somatic Chromosomes of Rats, *Cytologia*, **18**, 343, 1953.
57) Hauschka, T. S., Relationship between Chromosome Ploidy and Histocompatibility in Mouse Ascites Tumors, *Cancer Res.*, **12**, 269, 1952.
58) Hauschka, T. S. and Levan, A., Inverse Relationship between Chromosome Ploidy and Host-Specificity of Sixteen Transplantable Tumors, *Exper. Cell Res.*, **4**, 457, 1953.
59) Hauschka, T. S., Kvedar, B. J., Grinnell, S. T. and Amos, D. B., Immunoselection of Polyploids from Predominantly Diploid Cell Populations, *Ann. N. Y. Acad. Sci.*, **63**, 683, 1956.
60) Makino, S., Cytological Studies on Cancer, III. The Characteristics and Individuality of Chromosomes in Tumor Cells of the Yoshida Sarcoma with Contribute to the Growth of the Tumor, *Gann*, **43**, 17, 1952.
61) Makino, S., The Chromosome Cytology of the Ascites Tumors of Rats, With Special Reference to the Concept of the Stem Line Cell, *Internt. Rev. Cytol.*, **6**, 26, 1957.
62) Haldane, J. B. S., The Amount of Heterozygosis to Be Expected in an Approximately Pure Line, J. Genetics, **32**, 375, 1936.

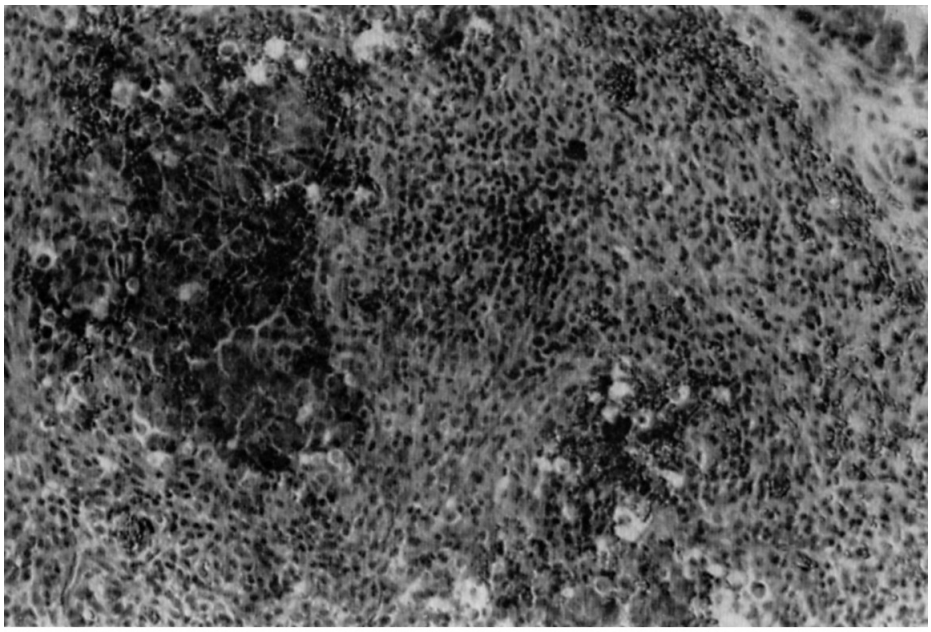


FIGURE 1. Section of the irradiated hamster tumor (first stage of selection). The tissue was stained with hematoxylin and eosin.

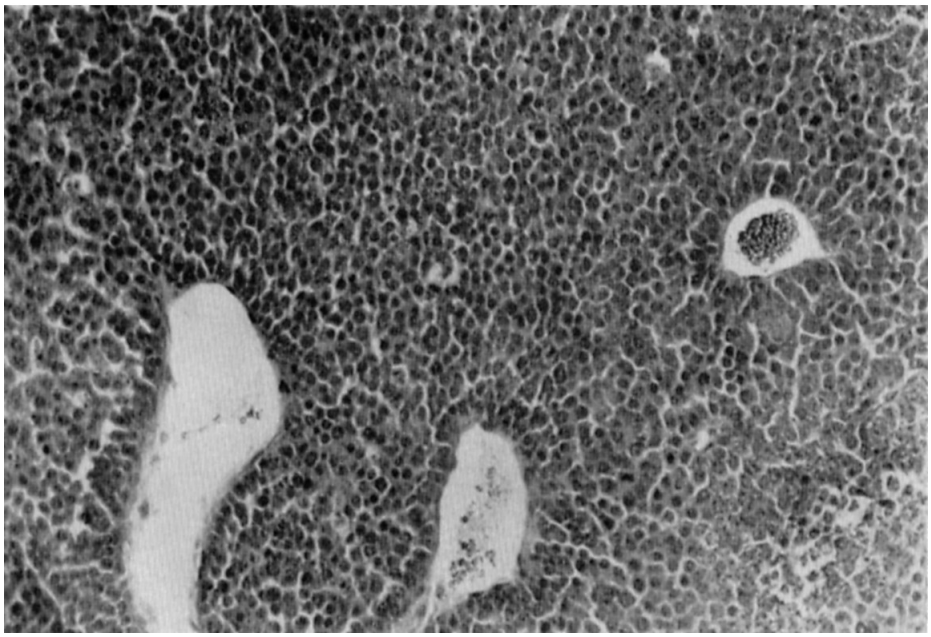


FIGURE 2. Section of the A×C rat tumor produced by the original H 4-II-E cells. The tissue was stained with hematoxylin and eosin.

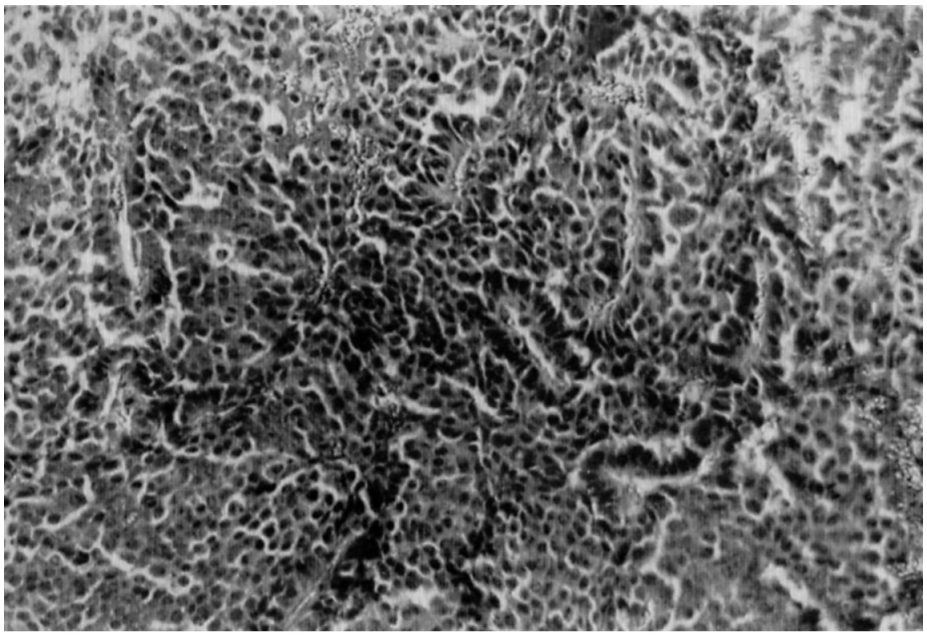


FIGURE 3. Section of the A×C rat tumor produced by the third selection explant #1. The tissue was stained with hematoxylin eosin.

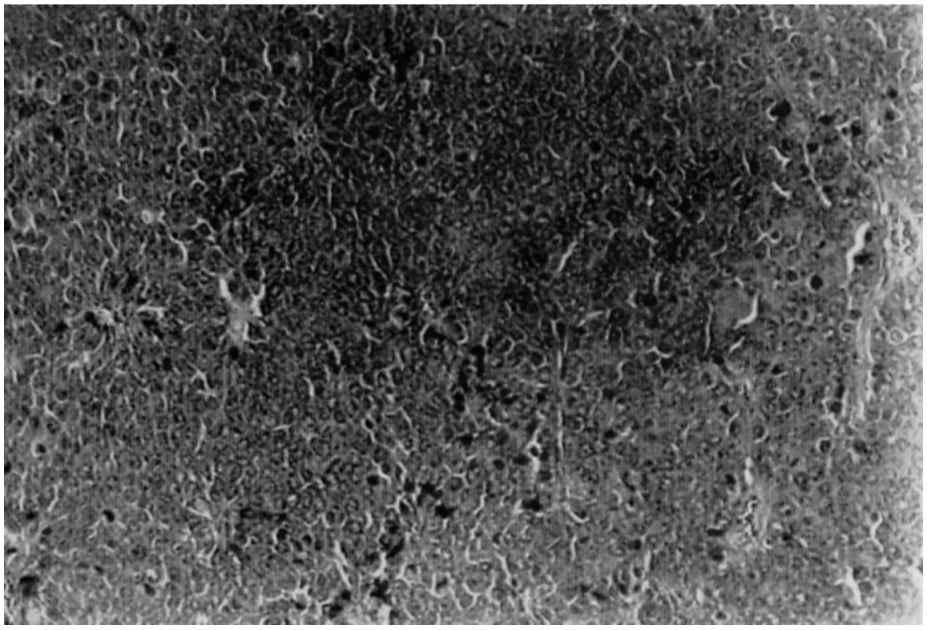


FIGURE 4. Section of the A×C rat tumor produced by the third selection explant #2. The tissue was stained with hematoxylin eosin.