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Studies on histocompatibility mutations in mouse tumour cells using isogenic strains of mice

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INTRODUCTION

The study of chemical mutagenesis in mammals has been limited for a number of reasons. The long interval between generations and the technical difficulties of testing large numbers of animals for quantitative mutation work make the study of mutations in higher organisms almost impossible. The treatment with mutagens of warm-blooded animals, e.g. mice, at doses high enough to produce genetical effects usually also produces severe systemic effects which make the tests of the compounds difficult. Most of these difficulties are overcome by testing for mutagenesis at the cellular level, either *in vitro* or *in vivo*. This is made possible by the use of tissue culture and tissue transplantation.

Tissue culture has been used fairly extensively to study the genetics of somatic mammalian cells *in vitro* (Puck, 1957, 1958*a*, *b*; Puck & Marcus, 1955; Puck & Fisher, 1956; Puck & Cieciura, 1958; Puck *et al.*, 1956, 1957). Tissue culture, while having certain advantages for quantitative work, is not ideal for chemical mutagenesis as the physiological environment in which the cells are growing is completely different from that found *in vivo*. Tissue transplantation, especially of neoplastic cells, is extremely useful. The transplantation of neoplastic cells in compatible hosts provides a condition in which the cells can divide rapidly and yet maintain their genetical stability over a number of generations. The availability of histocompatibility markers in mouse neoplastic cells provides a useful material for studying mutagenesis in these cells.

The development of isogenic resistant (IR) strains of mice by Snell (1948, 1953, 1955) provides a useful marker in studying cellular variation in mouse tumour cells. An IR strain of mice is genetically similar to the strain from which it is derived except for the histocompatibility-2 (H-2) locus. A tumour originating in a F_1 hybrid between two IR strains is homozygous at all loci, except the H-2 locus. The F_1 tumour is not transplantable to either of the parental strains. However, a single mutation at the H-2 locus towards one of the parental strains or a loss of one of the H-2 alleles in a hybrid tumour cell would make it compatible with that parental strain. It is, thus, theoretically possible to detect a single mutation occurring in a single cell of a large neoplastic population. Such a system was proposed by Lederberg (1956) and used by Mitchison (1956), Klein, Klein &

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Révész (1957) and Klein & Klein (1958, 1959). The aim of the present study is to see the feasibility of using such a system to test various mutagens and carcinogens for their mutagenic activity in mouse tumour cells.

Tumours induced in F_1 hybrids were tested in the parental strains to see if they would produce variants compatible with the parental strains. Hybrid tumours which produced variants regularly in the parental strains were tested after treatment with X-ray or triethylenemelamine to see if the production of variants could be increased by using these mutagenic agents. An increase in variant production by known mutagens would support the hypothesis that these variants are due to mutations at the H-2 locus.

MATERIALS AND METHODS

Mice

The strains of mice used were Snell's A strain and its two isogenic resistant sublines A.SW and A.CA, obtained from Prof. G. Klein, Karolinska Institutet, Stockholm. All three strains have a strain A background and were developed by Snell (1948, 1953, 1955). Snell's original A line is genotypically H-2a/H-2a, while its isogenic resistant sublines, A.SW and A.CA, are H-2s/H-2s and H-2f/H-2f, respectively (Snell, 1955; Allen, 1955). For convenience the H-2 genotype of the three strains will be referred to as a/a, s/s and f/f.

Tumours

Eighteen sarcomas were induced in hybrid mice (A × A.SW, H-2 genotype a/s) with 20-methylcholanthrene. The tumours were maintained by serial passage in hybrid mice. Most of the tumours were frozen in four parallel tubes, either immediately after the origin of the tumour or after a few passages in hybrid mice, and stored at −79°C. The tumours were numbered as.1, as.2, etc., indicating the H-2 genotype of the hybrid tumour. The tumours were transplanted subcutaneously by trocar and canula into the right flank or bilaterally.

To inject a known cell dosage, a suspension of the tumours in Ringer's solution was prepared by pressing them through a 60-mesh stainless steel sieve.

The cells were counted in a Buerker haemocytometer. The live cells were differentiated from dead cells by staining with 1% Nigrosin in Tyrode solution (Kaltenbach, Kaltenbach & Lyons, 1958). Cell counts were based only on unstained cells.

Treatment of cell suspensions with triethylenemelamine (TEM) and X-rays

Cell suspensions prepared as above were divided into two equal parts. One part was left standing at 3°C. while the other was subjected to the treatments.

The tumour cell suspensions were treated with TEM dissolved in sterile water at concentrations of 10 γ/c.c. (1:100,000) or 20 γ/c.c. (1:50,000). The treatments were done for 2 hours at 3°C. After treatment the cells were centrifuged and washed twice with Ringer's solution.

Cell suspensions were X-irradiated in pyrex centrifuge tubes at a distance of 250 mm. from the X-ray tube. The X-rays were generated at 140kV and filtered through 1 mm.Al. Doses of 150 r to 500 r were administered at a rate of 56·92 r per minute.

After treatment the cells were recounted in a Buerker haemocytometer after staining with 1% Nigrosin, and were brought to the required concentration. The percentage of unstained to stained cells varied between 15 and 25. It was found that the percentage of unstained cells to stained cells did not change after treatment with TEM or X-rays.

Mice inoculated with tumours were palpated regularly every third day after inoculation. Records of regression of the tumours were kept. Mice which failed to produce tumours were left for 3 months before they were killed and scored as negatives.

Cytological preparation of tumours

The tumours were prepared for cytological examination according to the method of Bayreuther & Klein (1958). The prepared pieces of tumour were stored in 45% acetic acid at −15°C. Permanent preparations were made by squashing small pieces of tumour on an albuminized slide and passing them through the alcohol series and mounting in Xam.

RESULTS

Transplantation tests with hybrid tumours ($A \times A.SW$ F_1, genotype a/s) induced with 20-methylcholanthrene

Fourteen hybrid tumours (H-2 genotype a/s) were tested in various numbers of parental mice, usually after the first transfer generation (t.g.). Some tests had to be delayed till after a few passages in hybrid mice. The results of the pooled transplantation tests are summarized in Table 1. These agree with those of Klein & Klein (1958, 1959). There is a large amount of variation in transplantation behaviour between individual tumours. It is, therefore, necessary to select carefully the tumours which are to be used in mutagenesis experiments.

Seven tumours behaved according to expectation: they grew only in the hybrid mice in which the tumour originated and gave no variants towards either of the parental strain.

Five tumours (as.1, 6, 7, 14 and 16) gave variants, occasionally or regularly, towards one or both of the parental strains. Table 2 gives the results of the transplantation tests with various variants that were isolated from hybrid tumours transplanted into parental mice. The variants bear the number of the original tumour followed by the number of the variant and the strain in which it originated. A.SW strain is designated as SW and strain A.CA as CA. In the production of the variants, the tumours always regressed completely at about 10–14 days after inoculation, and the variants then began to appear between one and two months after inoculation. Some of the tumours gave 'true variants' while others

gave 'false variants' which grew in mice of foreign genotypes and failed to grow in pre-immunized mice (see Klein & Klein, 1959).

Tumour as.7 was found to be the most interesting one and was extensively studied. It grew in 35·3% of A.SW mice and failed to grow in mice of the other parental strain or mice of foreign genotypes (Table 1). Six independent A.SW

Table 1. *Transplantation tests with various sarcomas induced by 20-methylcholanthrene in A × A.SW F_1 hybrids (H-2 genotype a/s)*

Host strain (and H-2 genotype)

Number of mice killed by tumour/Total number inoculated

Tumour no. and genotype	A × ASW F_1 (a/s)	A(a/a)		A.SW(s/s)		A.CA (f/f)	Other unrelated strains
		Untreated	Immunized	Untreated	Immunized		
as.1	22/22	1/22	0/16	2/22	0/24	0/6	0/12
2	0/6	0/10	—	0/12	—	—	—
3	12/12	0/10	0/10	0/13	0/26	—	—
4	12/12	0/12	0/12	0/10	0/10	—	—
5	12/12	0/24	—	0/24	—	—	—
6	10/10	0/10	—	1/12	—	—	—
7	96/96	0/81	0/24	36/102	2/26	0/20	0/10
8	11/11	0/12	—	0/12	—	—	—
9	11/11	0/13	0/12	0/12	0/12	—	—
10	12/12	—	—	0/12	—	—	—
14	14/14	2/12	—	5/12	—	0/12	—
15	6/6	0/12	—	0/12	—	—	—
16	13/13	1/6	—	0/10	—	—	—
17	14/14	0/18	0/6	0/14	0/6	—	—

variants of this tumour originating at different times were tested. They grew in 100% of the untreated and pre-immunized A.SW mice while failing to grow in A or A.CA mice (Table 2). One of the variants grew in 10/13 A × A.SW F_2 mice while another variant grew in 23/28 A × A.SW F_2 mice. Only one variant (as.7/2SW) produced a single growth in A.CA mice. This was found to be completely non-specific on further testing (Table 2). Tumour as.7 was also tested in 26 A.SW mice pre-immunized against this tumour. It grew only in 2 out of 26 (7·7%) of the pre-immunized mice.

Tumour as.14 was the only tumour that gave true variants towards both the parental strains, A and A.SW. The A variants grew in 100% of pre-immunized A strain mice and failed to grow in A.SW mice or mice of foreign genotypes. The A strain variants have remained specific over three transfer generations, thus showing them to be true variants for the A strain. The A.SW variant also failed to grow in mice of the other parental strain or of foreign genotypes and grew in a high proportion (13/18) of pre-immunized A.SW mice (Table 2).

Tumour as.2 failed to grow in mice of the parental strains as well as the hybrid mice in which the tumour originated. In all the mice inoculated, this tumour regressed after about 10 days, suggesting that the regression was due to the homograft reaction of the host. It is, therefore, possible that a mutation towards a

Table 2. *Transplantation tests with various variants originating from hybrid sarcomas (H-2 genotype a/s)*

Host strain (and H-2 genotype)
Number of mice killed by tumour/Total number inoculated

	A(a/a)		A.SW(s/s)					
Variants	Untreated	Immunized	Untreated	Immunized	A.CA (f/f)	Other unrelated strains	A × A.SW F_2	A.SW × A.CA (s/f)
as.1/1A	18/8	0/16	6/6	—	10/12	—	—	—
as.1/1SW	0/10	—	14/14	12/13	0/11	—	11/16	—
as.1/CA*	—	—	4/4	—	8/9	4/8	—	—
as.6/1SW	1/12	—	8/8	5/7	0/14	—	—	—
as.7/1SW	0/18	—	15/15	6/6	0/13	0/6	10/13	5/5
as.7/2SW	0/12	—	9/9	7/7	1/6	—	—	—
as.7/3SW	0/12	—	4/4	6/6	0/12	—	—	—
as.7/6SW	0/10	—	2/2	6/6	0/6	—	—	—
as.7/8SW	0/10	—	4/4	6/6	0/12	—	23/28	—
as.7/10SW	0/12	—	2/2	6/6	0/6	—	—	—
as.7/CA†	4/6	—	—	—	6/6	6/8	—	—
as.14/1A	2/2	12/12	0/6	—	0/6	0/5	—	—
as.14/1SW	0/8	—	2/2	13/8	0/7	0/5	—	—
as.16/1A	2/2	3/6	6/6	—	5/6	—	—	—

* Variant from A.CA host inoculated with variant as.1/1A.
† Variant from A.CA host inoculated with variant as.7/2SW.

different H-2 allele had taken place, most likely in the mouse in which the tumour originated.

Three of the original tumours, as.1, as.4 and as.7, became completely non-specific in the course of their passage in hybrid mice. The behaviour of these tumours after losing transplantation specificity is summarized in Table 3. In contrast to true variants originating in test mice, non-specific tumours failed to regress completely, although occasionally their growth remained retarded between 10 and 20 days after inoculation. Except for tumour as.4, the non-specific tumours failed to grow in pre-immunized mice. All these non-specific tumours were discarded in the study of the production of true variants towards the parental strains. In the case of tumour as.7, a subline derived from 10^2 cells at its fifth transfer generation in A × A.SW F_1 mice was used instead. This subline has been carried

Table 3. *Transplantation tests with three hybrid (A × A.SW F_1) sarcomas that became non-specific after a number of transfer generations (t.gs.) in A × A.SW F_1 mice*

Host strain (and H-2 genotype)
Number of mice killed by tumour/Total number inoculated

Tumour no. and genotype	No. of t. gs. in A × ASW F_1	A(a/a)		A.SW(s/s)		A.CA (f/f)	Other unrelated strains
		Untreated	Immunized	Untreated	Immunized		
as.1	5	9/12	0/16	0/16	0/18	5/6	0/5
4	4	22/22	6/6	20/20	4/4	12/12	—
7	7	15/31	0/6	16/26	0/6	11/13	4/5

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to its tenth transfer generation in hybrid mice and has remained very specific, growing only in hybrid A × A.SW F_1 mice, giving approximately 30% variants towards the A.SW strain and failing to grow in A and A.CA strains or strains of foreign unrelated genotypes.

Titration of mouse sarcoma cells for viability after treatment with triethylenemelamine (TEM) or X-rays

As a preliminary to studying the mutagenic effects of various physical and chemical mutagens on tumour cells, it is necessary to work out a proper dosage for treating the cells. This could be done by working out the survival of the tumour cells at various doses of treatment. Survival or viability tests are also essential for calculating mutation rates per surviving cells rather than per treated cells.

Survival of treated tumour cells was calculated by titrating serial tenfold dilutions of the untreated and treated cells in groups of compatible hybrid mice. Four to eight mice were used per dilution. This meant the use of large numbers of mice. As an alternative the chorioallantoic membrane (CAM) of a developing chick embryo was used. Grafts of mouse tumours are known to grow on the CAM until the eighteenth day of incubation (Murphy, 1912).

For CAM titrations, embryos at the tenth day of incubation were used, and 0·1 c.c. of the cell suspension was inoculated on the CAM close to a large blood-vessel. The CAMs were cut open and spread out on a Petri dish. They were examined under a binocular microscope against a dark background. Membranes with distinct tumours were counted as positive while membranes in which no tumours could be seen under the binocular microscope were considered as negatives.

The TD_{50} (50% end-point) for the untreated and treated series was calculated according to the method of Reed & Muench (1938). Table 4 gives the TD_{50} for the untreated, X-irradiated and TEM-treated cell suspensions obtained by titrating in mice and on the CAM of chick embryos. In Text-fig. 1 the log TD_{50} is plotted against the X-ray dose. The values obtained from titration on mice fall on a straight line. A lower TD_{50} value has been obtained in most experiments from CAM titrations than from mouse titrations. This could be due to the failure to distinguish true tumours from non-specific lesions (which also appeared on control membranes without any inoculation). Non-specific lesions are due to the damage produced

Table 4. *TD_{50} for the untreated, X-irradiated and TEM-treated cell suspensions of tumours as.7 and as.1 titrated in mice and on the CAM of chick embryos*

Tumour no. and genotype	Titration on	Untreated	X-irradiated		TEM-treated	
			150 r	500 r	10γ	20γ
as.7	Mice	$10^{2\cdot35}$	$10^{3\cdot07}$	$10^{4\cdot75}$	—	—
as.7	Mice	$10^{2\cdot31}$	—	—	$10^{3\cdot25}$	$10^{3\cdot71}$
as.7	CAM	$10^{1\cdot83}$	—	$10^{3\cdot64}$	—	—
as.7	CAM	$10^{2\cdot46}$	$10^{3\cdot33}$	—	—	—
as.7	CAM	$10^{1\cdot92}$	$10^{2\cdot56}$	$10^{4\cdot25}$	$10^{2\cdot54}$	—
as.1	CAM	$10^{2\cdot17}$	$10^{3\cdot22}$	—	—	—

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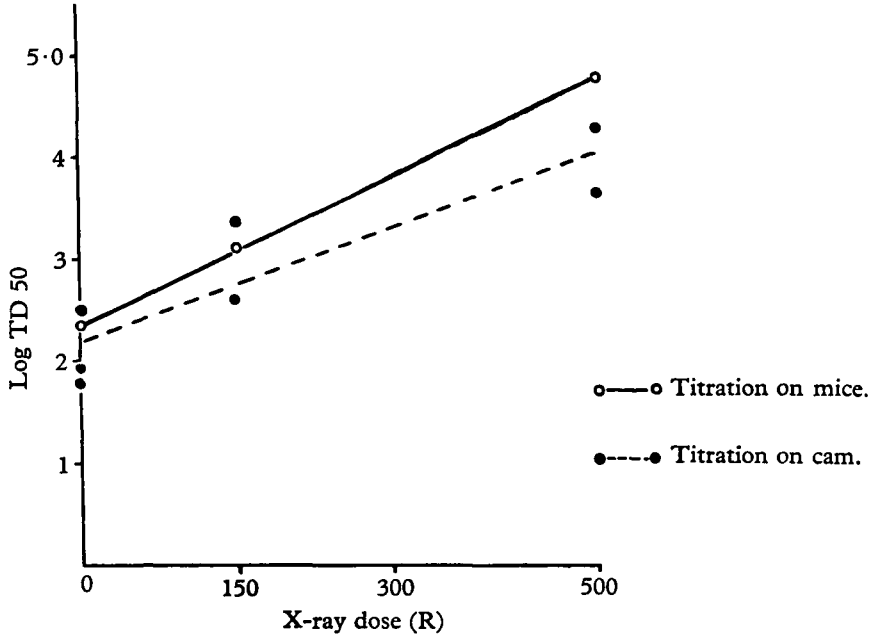
on the CAM or are a reaction on the part of the membrane to bits of shell dropping on it during inoculation. Titrations in mice appear to give better and more consistent results than titrations on CAM.

It must be noted that in all the experiments the TD_{50} for untreated tumour cell suspension lies in the region of 10^2 cells. In the untreated titration on mice no tumours were produced in twenty-four mice inoculated at a dilution of a single cell. One tumour was produced out of fourteen mice inoculated at a dilution of 10 cells. This suggests that a single viable cell is unlikely to give rise to a solid tumour when inoculated subcutaneously.



Text-fig. 1. Relationship between X-ray dose and log TD_{50} obtained by titrating tumour cell suspensions on mice and CAM.

The number of cells inoculated in the present case is based on the number of unstained cells. Recently, there have been a number of reports suggesting that not all stained cells are metabolically dead or inviable (B.E.C.C. Annual Report, 1951; Kaltenbach, Kaltenbach & Lyons, 1958). While the proportion of unstained cells could be used as an indication of the dilution range that should be titrated, the final results are best based on the total cell count.

Transplantation tests with 20-methylcholanthrene-induced hybrid sarcomas after treatment with triethylenemelamine and X-rays

Some of the hybrid tumours were selected in order to study the effects of triethylenemelamine (TEM) and X-rays on the transplantation behaviour of these tumours. These included tumours which were specific for the hybrid mice and

which gave variants regularly towards one of the parental strains. TEM and X-rays are the most powerful mutagens known and, as a preliminary, the investigation was limited to these two mutagens.

Cell suspensions of the tumours were prepared and treated with TEM and X-rays as described earlier. X-ray doses of 150 r, 250 r and 500 r were administered. TEM treatment was done at a concentration of 1 in 100,000 or 10 γ of TEM/c.c. of cell suspension for 2 hours at 3°C. The untreated controls and treated cell suspensions were inoculated into mice at the same time.

Table 5 summarizes the results of transplantation tests with tumour as.7 after treatment with TEM or X-rays in five different experiments. Tumour as.7, as already described, gave variants which were compatible with the A.SW parental strain but failed to give any variants towards the other parental strain (A). In experiments I to IV the cell suspensions were treated with TEM or X-rays *in vitro*, while in experiment V the tumour was treated with TEM *in vivo*. In experiments I to IV the untreated cell suspension of the tumour gave variants towards the A.SW strain in a percentage of mice ranging from 34·6 to 41·7. The average for the four experiments is 36·8%. The untreated cell suspensions of tumour as.7 failed to give any variants towards the A strain in all the experiments.

In experiments I to IV aliquots of the same cell suspension of tumour as.7 kept as untreated controls were treated with TEM at a concentration of 1 in 100,000 or 10 γ of TEM/c.c. of cell suspension. With the exception of experiment I, all experiments gave comparable results (Table 5). The percentage of variants isolated in the parental strain A.SW was significantly increased in the TEM treated series over the parallel untreated controls. The percentage of variants produced after treatment with TEM ranged from 57·1 to 61·5 for the three experiments, while the range for the untreated controls in the same three experiments was 34·6% to 37·5%. In experiment I, although there was no increase in the percentage of variants produced in A.SW mice per treated cells, there was obviously an increase per surviving cells after treatment. This experiment was also unique in that variants were produced after treatment with TEM towards the other parental strain in 4 out of 13 mice in which the tumour was tested (Table 5).

In experiments III and IV (Table 5) cell suspensions of tumour as.7 were treated with X-rays at a dose of 150 r for experiment III and 250 r for experiment IV. Again, the percentage of variants produced towards the A.SW strain was significantly increased after X-irradiation above those produced by untreated cell suspensions. In experiment III the percentage of takes in A.SW mice was increased from 35·7 with untreated cell suspension to 50·0 after treatment with 150 r X-rays, while in experiment IV the percentage of takes in A.SW mice was increased from 34·6 in the untreated series to 61·1 after treatment with 250 r X-rays. It thus appears that treatment with X-rays *in vitro* increases the number of variants produced and that 250 r is more effective than 150 r. No variants were produced in the A strain after treatment with 150 r or 250 r X-rays.

The above results with tumour as.7 show that TEM treatment and X-irradiation are effective agents in increasing the percentage of variants. Comparable cell

Table 5. *Transplantation tests with tumour as.7 without treatment and after* in vitro *treatment with TEM and X-rays in experiments I–IV. In experiment V the treatment with TEM was done* in vivo. *In parentheses are given the percentage of mice killed by progressively growing tumours*

Host strain (and H-2 genotype)

Number of mice killed by tumour/Total number inoculated

Expt. no.	Untreated			X-ray-treated			TEM-treated		
	Cell dosage	A(a/a)	A.SW(s/s)	Cell dosage	A(a/a)	A.SW(s/s)	Cell dosage	A(a/a)	A.SW(s/s)
I	$3{\cdot}5 \times 10^6$	0/12	5/12 (41·7)	—	—	—	$2{\cdot}5 \times 10^6$	4/13 (30·8)	4/11 (36·4)
II	$3{\cdot}8 \times 10^5$	0/28	9/24 (37·5)	—	—	—	$3{\cdot}0 \times 10^5$	0/24	6/10 (60·0)
III	$9{\cdot}6 \times 10^5$	0/13	5/14 (35·7)	$9{\cdot}6 \times 10^5$*	0/14	7/14 (50·0)	$7{\cdot}6 \times 10^5$	0/11	8/13 (61·5)
IV	$6{\cdot}3 \times 10^5$	0/28	9/26 (34·6)	$6{\cdot}3 \times 10^5$†	0/28	11/18 (61·1)	$6{\cdot}1 \times 10^5$	0/14	8/14 (57·1)
Total for expts. I–IV		0/81	28/76 (36·8)		0/42	18/32 (56·3)		4/62 (6·45)	26/48 (54·2)
V	$2{\cdot}9 \times 10^5$	0/12	4/12 (33·3)	—	—	—	$2{\cdot}9 \times 10^5$	0/12	0/12

* X-ray dose = 150 r.
† X-ray dose = 250 r.

doses were inoculated in the untreated controls and the treated series. Hence, both TEM and X-rays are effective in increasing the percentage of variants per treated cells. A proportion of the cells are killed as a result of the treatment. To estimate the actual effectivity of the agents used a correction has to be made for this. However, this correction is not a straightforward one in the above case as both the compatible and incompatible cells among the survivors and the untreated controls divide an unknown number of times before the homograft reaction sets in and selectively destroys the incompatible cells.

As the main interest in the present study was in chemical mutagens, only variants isolated in strains A and A.SW after treatment with TEM were tested further. The behaviour of these variants is summarized in Table 6. Three variants from each strain were tested. Two variants isolated in A.SW mice were specific for strain A.SW, growing in untreated A.SW as well as a high percentage of pre-immunized A.SW mice, and failing to grow in the other parental strain or mice of

Table 6. *Transplantation tests with variants of tumour as.7 isolated in strains A and A.SW mice after treatment* in vitro *with TEM*

Host strain (*and H-2 genotype*)

Number of mice killed by tumour/Total number inoculated

	A(a/a)		A.SW(s/s)		A.CA	Other unrelated
Variant	Untreated	Immunized	Untreated	Immunized	(f/f)	strains
as.7/4SW	0/12	—	6/6	5/9	0/6	—
as.7/5SW	0/12	—	4/4	5/6	0/12	0/6
as.7/7SW	—	—	4/4	6/6	2/12	1/6
as.7/1A	9/9	6/6	0/6	—	0/12	—
as.7/2A	10/10	5/6	3/6	—	0/12	—
as.7/3A	4/4	6/6	4*/12	—	—	0/6

* One of the four tumours was tested further in seven A.CA mice and grew in five of them.

foreign genotypes (A.CA and unrelated strains). The other variant gave a proportion of takes (2/12) in A.CA mice and in mice of unrelated strains (1/6). Three of the four variants produced in strain A after treatment with TEM were tested. Only one of these three variants was found to be specific for the A strain (Table 6). It failed to grow in the other parental strain A.SW and grew in pre-immunized mice of the A strain. The second variant grew in a proportion (3/6) of the A.SW mice but in none of the A.CA mice (0/12). This variant was, therefore, not completely specific for A strain. The third variant gave 4 tumours out of 12 mice of strain A.SW in which the variant was tested. One of the four tumours was further tested in A.CA mice and was found to grow in 5 out of 7 mice. This variant appears to be non-specific to a large extent.

In experiment V (Table 5) the tumour cells were treated *in vivo* with TEM as follows. Four hybrid mice (A × A.SW F_1) were inoculated with tumour as.7. After 10 days when the tumours were of reasonable size, two mice were injected intraperitoneally with a solution of TEM at a dose of 2 mg./kg. body weight. Two inoculations of TEM were done on two consecutive days. Two mice with

tumours were left untreated. Twenty-four hours after treatment, the tumours from the treated and untreated hybrid mice were removed and tested in the parental strains as shown in Table 5. The percentage of unstained cells in tumours from the untreated mice was 28·5, while in tumours from the treated mice it was 15·6. The untreated tumours gave variants in 33·3% of A.SW mice and none in the A strain, while in contrast to treatment *in vitro* the treated tumours gave no variants in either of the parental strains. This could be due to the fact that very few viable cells were present in the treated tumours. Moreover, the tumours would receive an uneven treatment and parts of the tumour receiving an effective dose of TEM would also be the parts that would be killed by the chemical.

In order to detect if a hybrid tumour could be changed towards a new H-2 allele, tumour as.7 was tested after treatment with TEM or X-rays in hybrid mice between A and A.CA and A.SW and A.CA (Table 7). As was found by Klein & Klein (1958), no true variants, specific only to these hybrids, could be isolated.

Table 7. *The top part of the table gives results of transplantation tests with tumour as.7 in A × A.CA F_1 and A.SW × A.CA F_1 mice after treatment with TEM and X-rays. The bottom part gives the results of transplantation tests with the variants which were tested without any treatment*

Host strain (and H-2 genotype)

Number of mice killed by tumour/Total number inoculated

	A × A.CA F_1 (a/f)		A.SW × A.CA F_1 (s/f)	
Tumour	TEM-treated	X-irradiated	TEM-treated	X-irradiated
as.7	1/7	0/6	1/6	7/14
as.7/A.CA (variant from A × A.CA)	A(a/a) 0/10	A × A.CA (a/f) 0/6	A.SW(s/s) —	A.SW × A.CA (s/f) —
as.7/S.CA (variant from A.SW × A.CA)	—	—	6/6	7/7

In addition to tumour as.7, three other tumours were tested in the parental strains after treatment with TEM or X-rays. Two of these tumours (as.3 and as.17) were very specific and had given no variants towards either of the parental strains. They failed to give variants after treatment with TEM or X-rays (Table 8). Tumour as.1 had given variants earlier spontaneously towards the A.SW strain. This tumour failed to give any variants when irradiated with 500 r. This may be due to the high X-ray dose used which would kill a large proportion of the tumour cells.

Cytological analysis of hybrid (A × A.SW F_1) sarcomas and their derived variants

Cytological analysis was carried out on two of the original hybrid tumours, as.7, and their variants isolated in mice of the parental strains. Chromosome counts were made by drawing roughly each metaphase on a piece of paper and counting the number of chromosomes from the paper. Checks were made on each metaphase

Table 8. *Transplantation tests with three hybrid tumours without treatment and after treatment* in vitro *with X-rays and TEM*

Host strain (and H-2 genotype)

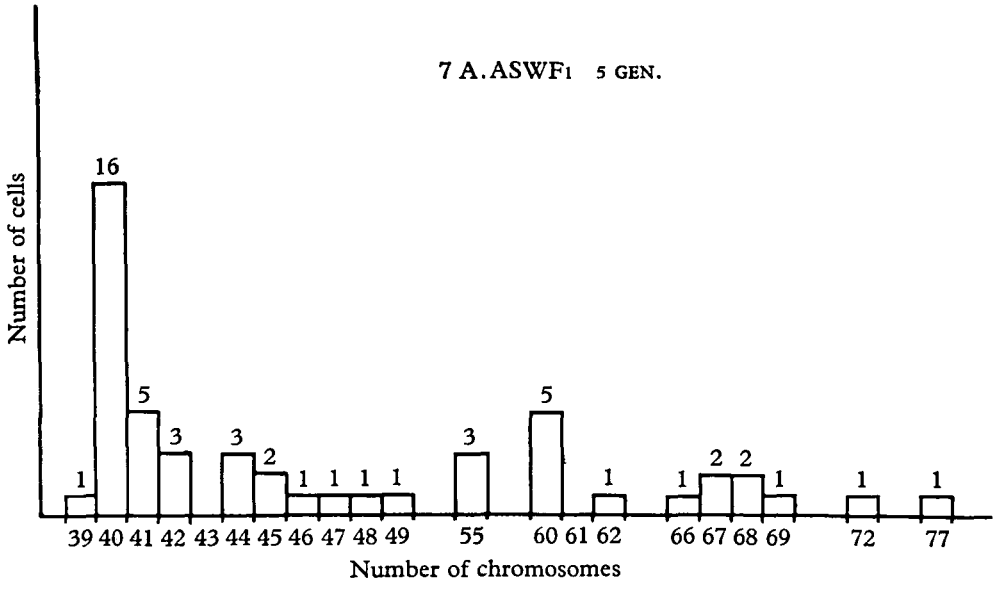
Number of mice killed by tumour/Total number inoculated

Tumour no. and genotype	Untreated			X-irradiated, 500 r			TEM-treated, 10 γ		
	Cell dosage	A(a/a)	A.SW(s/s)	Cell dosage	A(a/a)	A.SW(s/s)	Cell dosage	A(a/a)	A.SW(s/s)
as.1	$1{\cdot}8 \times 10^6$	1/22	2/22	$1{\cdot}8 \times 10^6$	0/24	0/24	—	—	—
as.3	$1{\cdot}7 \times 10^6$	0/10	0/13	$1{\cdot}5 \times 10^6$	—	0/11	—	—	—
as.17	$1{\cdot}1 \times 10^6$	0/8	0/14	—	—	—	$9{\cdot}0 \times 10^5$	0/13	0/12

by counting and recounting the chromosomes directly under the microscope with a tally counter. While the majority of the counts were exact, metaphase plates showing an error of more than ± 3 chromosomes were not included.

Tumour as.7

The cytology of tumour as.7 at its fifth transfer generation in hybrid (A × A.SW F_1) mice was examined. Fifty metaphases were examined and the chromosomes counted. As can be seen from Text-fig. 2, this tumour had a diploid modal number (40 chromosomes) as well as a few cells around the triploid number (60). Most of



Text-fig. 2. Chromosome number distribution of tumour as.7 at its sixth generation, based on fifty metaphases.

the cells counted were exactly diploid (Plate I, Fig. 1), while a few cells were also found to be hyperdiploid. A small number of cells were triploid or hypertriploid and only 2 cells out of 50 counted had a hypotetraploid number of chromosomes. It may be of significance to point out here that this subline of tumour as.7 became non-specific at its seventh transfer generation in hybrid mice. It is possible that this could be due to the triploid cells, which were already present as a minority at the fifth transfer generation of the tumour, replacing the modal diploid cells. No marker chromosomes were regularly encountered and hence no detailed idiograms were drawn.

Variant as.7/1SW

This variant was isolated from an A.SW mouse inoculated with tumour as.7 at its first transfer generation in hybrid mice. The variant was maintained by regular transplantation in A.SW mice. The cytology of this variant at its third

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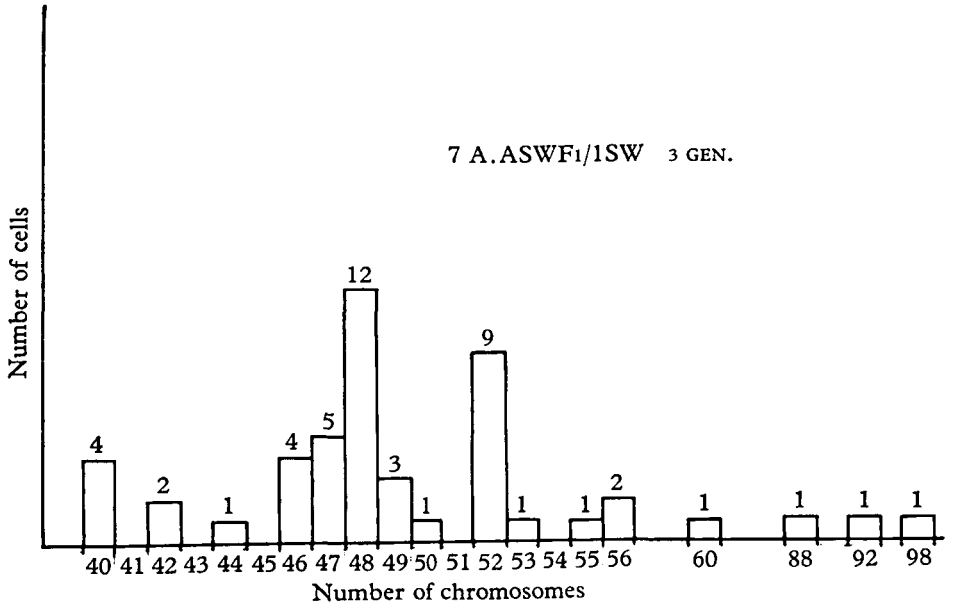
transfer generation was examined. The distribution of chromosome numbers based on fifty metaphases is given in Text-fig. 3. Compared with the original tumour there appears to be a shift in the modal number from exactly diploid in the original tumour to hyperdiploidy. There appear to be two modes—one at 48 chromosomes (Plate I, Fig. 2) and another one at 52 chromosomes. There are fewer cells at the triploid number of chromosomes compared with the original tumour. This could be due to the early isolation of the variant from the original tumour compared with when it was used for cytological examination.



Text-fig. 3. Distribution of chromosome number of the variant 1SW, third transfer generation, of tumour as.7 which originated spontaneously. Examination of fifty metaphases.

Variant as.7/4SW

This variant was derived from an A.SW strain mouse inoculated with a cell suspension of as.7 at its third transfer generation after treatment with TEM. Cytological examination of this variant was carried out after its second passage in A.SW mice. This variant also has two modal numbers of chromosomes—one at 42 and another at 60 (Text-fig. 4, Plate II, Fig. 3). Compared with variant as.7/1SW which originated spontaneously, this variant has a larger proportion of cells with chromosome counts approximating to the triploid number.

Variant as.7/2A

This variant was also isolated from tumour as.7 which was treated with TEM at its third transfer generation. The variant was not completely specific for the A strain (Table 6). Cytological analysis of the variant at its second transfer

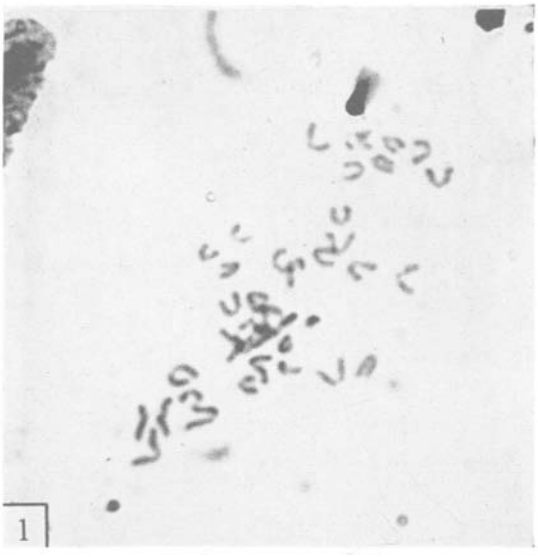


Fig. 1. Photomicrograph of a metaphase of tumour as.7, 5 t.g. with 40 chromosomes. × 1800.

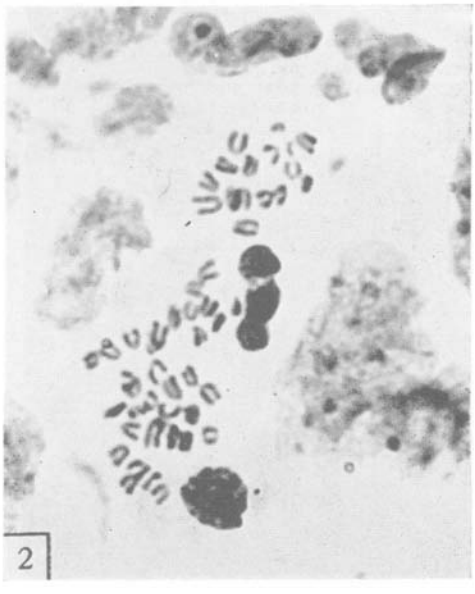


Fig. 2. Photomicrograph of a metaphase of variant as.7/1SW, 3 t.g. with 48 chromosomes. × 1800.

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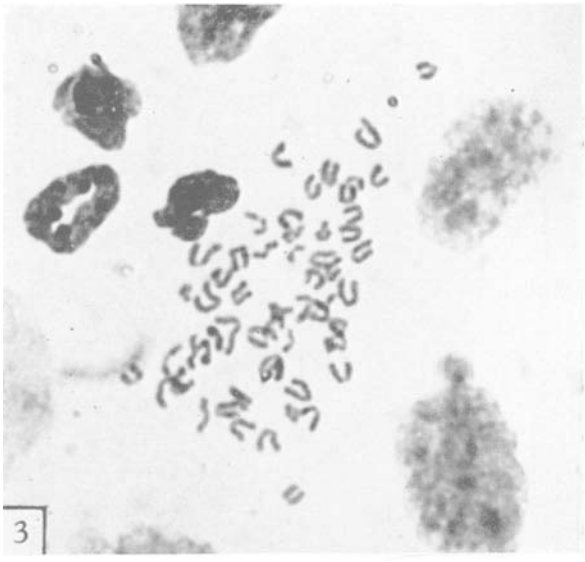

Fig. 3. Photomicrograph of a metaphase of variant as.7/4SW, 3 t.g. with 60 chromosomes. ×1800.

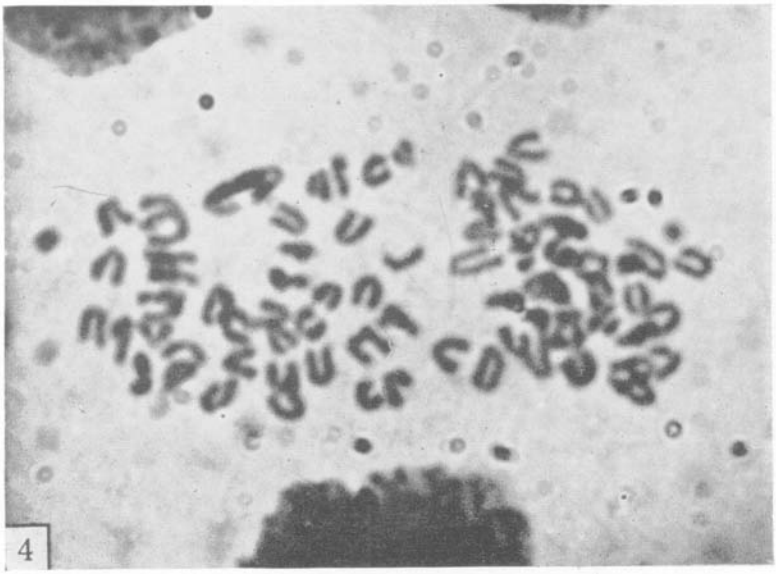

Fig. 4. Photomicrograph of a metaphase of variant as.7/2A, 2 t.g. with 72 chromosomes. ×2500.

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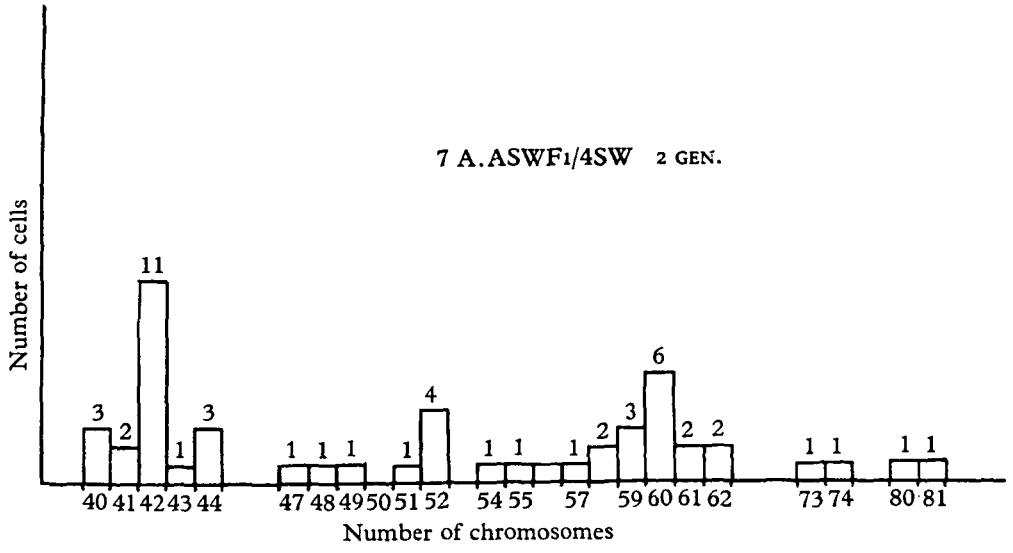
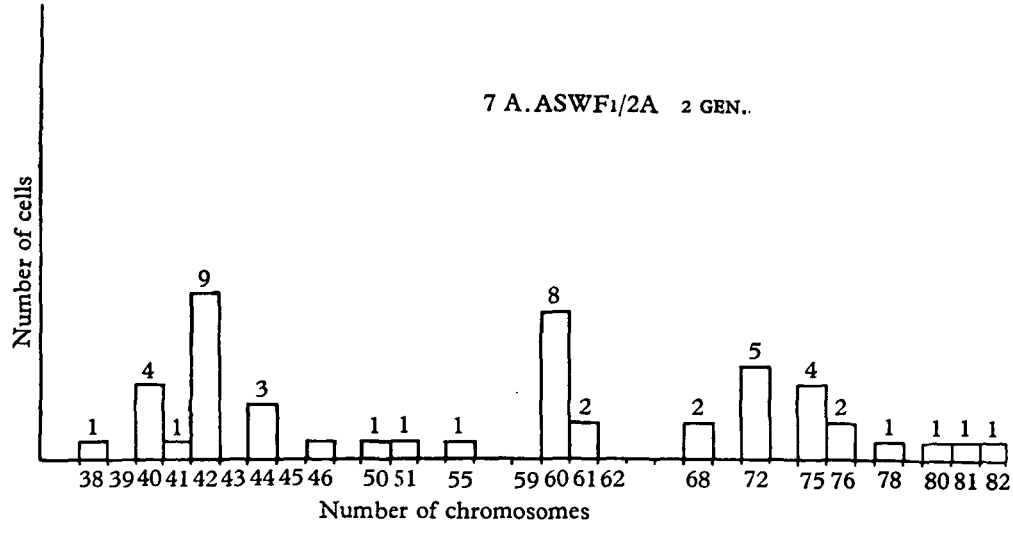


Text-fig. 4. Distribution of the chromosome number of the variant 4SW, 2 t.g., isolated after TEM treatment from tumour as.7, based on fifty metaphases.



Text-fig. 5. Chromosome number distribution of variant 2A, second generation isolated from tumour as.7, after TEM treatment. Fifty metaphases were examined.

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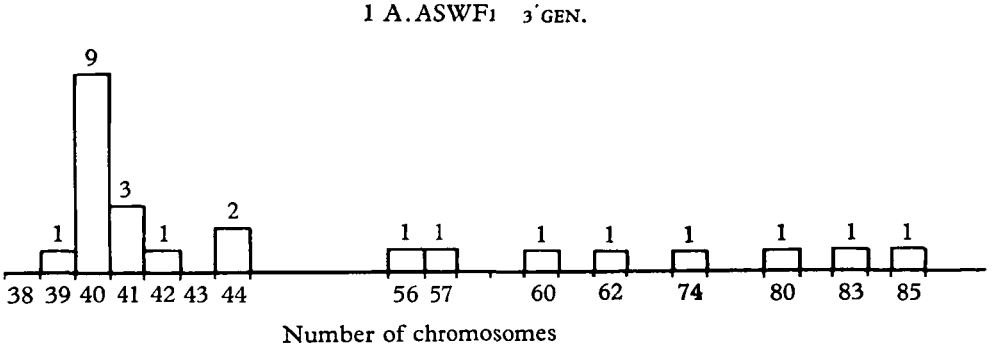
generation in A strain mice showed that it had almost equal numbers of cells with the hyperdiploid number (modal number 42) and with the triploid number of chromosomes. The chromosome number distribution for this variant based on fifty metaphases is shown in Text-fig. 5. It also contained a high proportion of cells at the hypotetraploid number (Plate II, Fig. 4). In accordance with the findings of Hauschka & Levan (1953), this would explain the incomplete specificity of this variant for the A strain.

As far as the hyperdiploid modal number of variants as.7/4SW and as.7/2A (both of which were isolated after TEM treatment) is concerned, there appears to have been a shift from 40 chromosomes in the original tumour to 42 chromosomes in the variants. Both these variants, compared with the original tumour and the spontaneous variant, have a larger proportion of cells around triploid and hypotetraploid numbers. This suggests that TEM induces polyploidy. This action of TEM has been demonstrated by Yerganian (1956) for the chromosomes of the Chinese hamster.

Tumour as.1

The cytology of this tumour at its third transfer generation in hybrid mice was examined. Only twenty-five metaphases were studied and chromosome counts made. Text-fig. 6 shows the chromosome number distribution for this variant. It has a modal number of diploid cells with occasional cells around the triploid and tetraploid numbers. No marker chromosomes again could be detected for this tumour and detailed idiograms were not prepared.

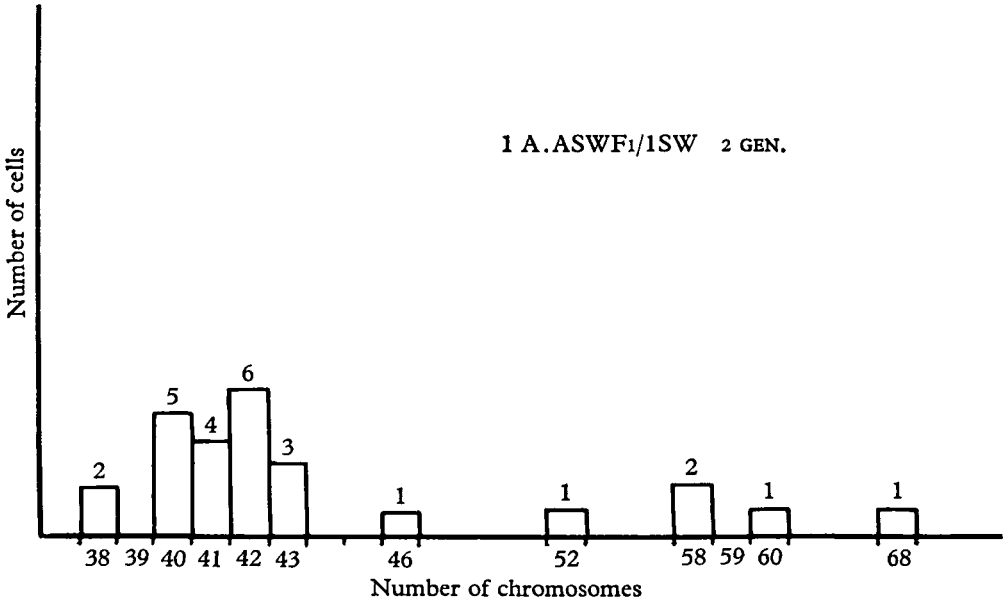


Text-fig. 6. Chromosome number distribution of tumour as.1 at its third generation, based on twenty-five metaphases.

Variant as.1/1SW

This variant was isolated in an A.SW mouse from tumour as.1 at its first transfer generation. The variant was specific for A.SW strain. Again twenty-five metaphases were examined after the variant had been passaged for two transfer generations in A.SW mice. The chromosome number distribution for this variant

is given in Text-fig. 7. Compared with the original tumour, this variant has an increased number of cells with hyperdiploid number of chromosomes, although there was not a complete shift towards hyperdiploidy—chromosome counts ranged from 38 to 43. A few cells with chromosome complements around the triploid number were present.



Text-fig. 7. Distribution of chromosome number in twenty-five metaphases of variant 1SW, 2 t.g., of tumour as.1, which originated spontaneously.

DISCUSSION

The aim of the present study was mainly to investigate the feasibility of using histocompatibility markers in isogenic strains of mice to study the effects of various mutagens on mammalian cells. Although the preliminary nature of the experiments need hardly be stressed, they indicate the advantages and disadvantages involved in using such a method.

Hybrid tumours induced in various combinations of isogenic strains of mice have been used to detect variants compatible with one of the parental strains (Mitchison, 1956; Klein, Klein & Révész, 1957; Klein & Klein, 1958, 1959). It has been shown by these workers that it is possible to obtain variants from heterozygous tumours selectively compatible with one of the parental strains. The results were compatible with the hypothesis that the variants were the result of a genetic change. While no direct evidence can be obtained in support of the occurrence of a mutation in somatic cells, a number of criteria can be used in support of it. If it is assumed that a mutation has taken place, response to mutagenic treatment would support the assumption.

One of the interesting features of variant production by the hybrid tumours is the asymmetry of response towards the two parental strains. With tumours of any hybrid combination one parental strain is always favoured for variant production (Klein & Klein, 1958, 1959). In the present investigation this asymmetry of response is also very marked. Tumour as.7 gave variants only in A.SW mice in the range of 33·3–41·7 % in a number of different experiments. This range is higher than that of MSWB tumour of Klein, Klein & Révész (1957), which gave variants in 15–25 % of A.SW mice (Klein & Klein, 1958). In contrast to this tumour, another tumour, as.14, gave variants specifically compatible with both the parental strains. Klein, Klein & Révész (1957) and Klein & Klein (1958) failed to get variants towards this parental strain with hybrid tumours of the cross A × A.SW. It thus appears that a hybrid tumour of the cross A × A.SW can give selectively compatible variants towards both the parental strains, although it still showed a preference for the A.SW strain.

As has been pointed out by Klein & Klein (1958), it is interesting to note that tumours induced in hybrids of the same inbred strains by the same dose of carcinogen in the same tissue should show such large differences in their transplantation behaviour. Most of the tumours were very specific while some gave variants towards one of the parental strains. Three of the tumours became non-specific after a number of passages in hybrid mice. As two of these tumours had given specific variants towards the A.SW strain before becoming non-specific, it may be postulated that the production of specific variants was a preliminary step to the tumours becoming non-specific and that the two phenomena are comparable. However, a subline of tumour as.7 which gave specific variants towards the A.SW strain and became non-specific in its seventh transfer generation was derived from 10^2 cells at its fifth transfer generation. This subline has remained specific to date, although still producing variants towards A.SW strain, and has been carried to its tenth transfer generation in hybrid mice. It is noteworthy that different sublines of the same tumour can change differently in their transplantation behaviour.

To estimate the mutagenic effectivity of mutagens, chemical or physical, it is essential to calculate the viability of the cells after treatment with the mutagenic agent. This is also necessary in order to establish a dose high enough to produce genetical effects without killing a large proportion of the cells. Staining of the tumour cell suspensions with 1 % Nigrosin in Tyrode solution was used to distinguish between alive and dead tumour cells (Kaltenbach, Kaltenbach & Lyons, 1958). It was found that the percentage of unstained cells to stained cells was the same after treatment with TEM or X-rays as before treatment. This shows that killing of the tumour cells by treatment with X-rays or TEM could not be detected by the staining technique. Vinegar (1956) using microfluorescence cell-viability test with acridine orange to differentiate dead and live cells in ascites tumours found that the test was successful in detecting death due to acid, base, ethanol, diphenylamine diazomium bromide, aerobic autolysis and ultrasonic radiation. He failed to detect tumour cell injury caused by X-rays, a nitrogen mustard and

bromide ion. Kaltenbach *et al.* (1958) were also able to increase the proportion of stained cells by treating ascites tumours with a hypotonic solution. It would appear, therefore, that staining of tumour cell suspensions can detect death due to cytotoxic agents. However, damage caused by X-rays and TEM cannot be detected, as the damage in this case and in the case of nitrogen mustard and bromide ions could be nuclear. The cell would thus need to divide before the damage could be detected. Viability of tumour cells after treatment with X-rays, TEM and possibly other mutagens could, therefore, only be detected by titration of the untreated and treated cell suspensions in groups of mice.

Viability of the tumour cells after treatment with TEM or X-rays was estimated by titrating the untreated and treated cell suspensions in groups of mice and on the chorioallantoic membranes (CAM) of groups of developing chick embryos. The TD_{50} values obtained by titrating cell suspensions on groups of mice or on the CAMs of groups of chick embryos gave comparable results although titration in mice gave more consistent results.

Titration experiments with hybrid sarcomas showed that approximately 10^2 untreated cells (the cell count being based on unstained, presumably viable cells) were needed to produce a tumour. This is actually an underestimate as the above results were based on unstained cells. It would appear that single cells fail to produce solid carcinomas or sarcomas. However, ascites tumours and leukemias have been conclusively proven to be transplantable with a single isolated cell. This has been demonstrated for the Yoshida rat ascites sarcoma (Ishibashi, 1950) and for the Ehrlich and Krebs 2 ascites carcinoma (Hauschka, 1953 *a*, *b*; Hauschka & Levan, 1958).

Although a single cell may be unable to produce a sarcoma in compatible mice, it does not follow that a single variant cell is unable to produce a variant when tested in the parental strains. In this case, the single compatible cell and the large number of incompatible cells undergo a number of divisions before the homograft reaction of the host selectively destroys the incompatible cells. It is possible, therefore, that a single variant cell which would undergo divisions and form a clone could then give rise to a variant tumour. This is substantiated to some extent by the experiments of Klein & Klein (1956 *a*). They obtained takes in mice where 4 compatible cells were mixed in 10^7 incompatible cells.

The percentage of variants produced is considerably decreased when the hybrid tumours are tested in pre-immunized parental mice. In the present investigation, tumour as.7 gave variants in only 7·7 % of pre-immunized A.SW mice compared with 35–40 % in untreated mice. Klein & Klein (1958) obtained only 2 % variants in pre-immunized A.SW mice with the MSWB tumour which gave 20–25 % variants in untreated mice. It has been suggested that the difference is due to the fact that in pre-immunized mice variant production is only due to the selection of pre-existing variant cells while in untreated mice a number of variant cells may be produced during the division of the incompatible cells. As the incompatible cells, when inoculated in pre-immunized mice, undergo no or very limited division before they are destroyed by the homograft reaction, it is possible that the lower

percentage of variants produced may be due to the requirement of a larger number of variant cells to produce a tumour. This would have to be checked by titrating mixtures of compatible and incompatible cells in untreated and pre-immunized or hyper-immunized mice.

Although the results obtained did not give quantitative estimates, it was established that the hybrid tumours do respond to mutagenic treatment. Treatment with TEM or X-rays of tumour as.7 increased the percentage of variants per treated cells towards the A.SW strain. With the exception of one experiment, no variants were produced towards the A strain after treatment. Only one of four variants isolated in the parental strain A after treatment with TEM was specific for this strain, while two others did not show complete specificity for the A strain. One of these variants grew in both A and A.SW mice, but failed to grow in mice of foreign genotypes. Variants isolated in A.SW mice after treatment with TEM showed greater lack of specificity for A.SW mice than the variants isolated spontaneously from the same tumour. It appears, therefore, that TEM treatment induces a certain proportion of non-specific variants. Cytological findings give some support to this.

The cytological analysis of the original tumours and variants isolated without and with treatment by TEM gave interesting results. It was found that the variants isolated without any treatment had a different modal number of chromosomes. While the original tumour line had a mode at approximately 40 chromosomes, the variants usually were hyperdiploid. The variants isolated after treatment with TEM gave different karyotypes compared with the untreated variant. While the original tumour and untreated variants had only a small proportion of triploid cells and very few hypo- and hyper-tetraploid cells, variants resulting after treatment with TEM had a large proportion of cells around the triploid and tetraploid number. A difference was also noted between the variants isolated after TEM treatment in A and A.SW strains. The A.SW variant had few cells around the tetraploid number while the A strain variant had a comparatively larger number of hypotetraploid cells. In agreement with the findings of Hauschka & Levan (1953), who found a correlation between tumour specificity and polyploidy of the cells, the above cytological findings would explain the incomplete specificity of the variants resulting after TEM treatment.

These results are in agreement with those of Bayreuther & Klein (1958) although the cytology of our variants was not examined in as much detail as in their work. They found that each independently isolated variant of MSWB tumour had a characteristic karyotype which was distinct from the original tumour. While the original tumour had a stemline at the diploid number, the variants were always hyperdiploid.

A definite increase in the frequency of variants produced is noted after treatment with X-rays or TEM. Variant production appears to be always associated with chromosomal derangements. In the case of TEM, the increase in variant frequencies appears to be partly due to its cytological effects. A certain percentage of non-specific variants are produced after TEM treatment due to the presence of larger numbers of polyploid cells.

The nature of the mechanism of variant formation in heterozygous tumours and the question whether these variants are due to genetical or non-genetical modifications, have been discussed by Klein & Klein (1959). From the fact that true-breeding variants are obtained with heterozygous but not with homozygous tumours, they conclude that the mechanism of variant formations is probably genetical or nuclear in origin.

At the genetic level, possible mechanisms include somatic crossing over, point mutations, deletions, loss of a chromosome or larger chromosomal changes, and transduction. These have been dealt with by Klein & Klein (1958, 1959) and need only be mentioned. Transduction, involving the change of genetic material between the host and tumour cells, appears to be unlikely as transduction has not yet been demonstrated for mammalian cells.

The use of heterozygous tumour cells is a prerequisite to studying mutations with histocompatibility markers as the probability of a double mutation occurring at the same locus is infinitesimally small. Hence, the possibility of somatic crossing over in heterozygous tumour cells cannot be ruled out.

The question whether variant formation could be due to point mutations at the histocompatibility locus is a difficult one. One objection to point mutation is that most of the isogenic resistant strains differ with regard to two or three antigenic components and it is unlikely that single point mutations could alter a number of antigenic components. However, Hoecker (1956) has suggested that a single genetical change may induce a multiple change of serological specificities.

The fact that all the variants have a hyperdiploid number of chromosomes suggests that duplication of some genetic material may play a role in variant formation. Sachs & Gallily (1956) have shown that not all the chromosomes have to be duplicated for a tumour to become non-specific. It may be postulated that duplication of a chromosome containing one H-2 allele may make a variant non-specific for that allele, whatever may be the mechanism of non-specificity in relation to chromosome duplication. This variant would then be specific for the other H-2 allele. Thus, duplication of the chromosome carrying H-2a would give a cell with the genotype H-2a/H-2a/H-2s, which would only be specific for the H-2s allele.

While variant formation in hybrid sarcomas appears usually to be associated with cytological changes, Hellström found variants among hybrid lymphomas where no cytological changes could be detected (Klein & Klein, 1959). This suggests that tumours other than sarcomas with more stable cytological constitutions may be more suitable for mutation studies with isogenic strains of mice.

SUMMARY

The feasibility of using heterozygous tumours induced in hybrids between Snell's isogenic resistant (IR) strains of mice for mutation studies was examined. The system has been described by Mitchison (1956) and Klein, Klein & Révész (1957).

Fourteen hybrid sarcomas (A × A.SW F_1; H-2 genotype a/s) were tested in mice of the parental strains. Seven were specific for the original F_1 hybrid genotype and failed to give any variants. Four tumours gave variants that were compatible with one of the parental strains. The variants grew progressively in all mice of the parental strain, even in pre-immunized mice, and failed to grow in mice of foreign genotypes. Of these, one tumour, as.14 gave true variants towards both the parental strains. One tumour, as.7, gave variants regularly in 35·5% of the A.SW mice but only in 7·7% of pre-immunized A.SW mice. It failed to give variants towards the other parental strain.

The effect of triethylenemelamine (TEM) and X-ray treatment on the rate of variant production in hybrid tumours was studied. The effect of the treatment on the viability of tumour cells was first examined by titrating tumour cell suspensions on groups of mice or on the CAMs of groups of developing chick embryos. The TD_{50} values (50% end-points) were calculated. Comparable results were obtained by titrating on mice and on the CAM, although titration on mice gave more consistent results. Approximately 10^2 viable, untreated cells were needed to produce a tumour in mice or on the CAM.

Tumour as.7 was tested in the parental strains after treatment with TEM or X-rays. Using comparable cell doses in the untreated controls and treated series, the percentage of variants produced in one of the parental strains (A.SW) was significantly increased after treatment with TEM or X-rays. With the exception of one experiment, no variants were produced in the other parental strain. In one experiment, four variants were produced towards the A strain after treatment with TEM, three of which were tested: only one was found to be specific for the A strain. Three of the A.SW variants, originating after treatment with TEM, were tested: two were specific while one showed incomplete specificity for the A.SW strain. Treatment of specific tumours with TEM or X-rays did not give variants towards either of the parental strains.

Two of the hybrid tumours and their derived variants were examined cytologically. Variants originating spontaneously and after treatment with TEM were examined. While most of the original tumours had chromosome numbers with an exactly diploid modal number, the spontaneous and TEM-treated variants had modal numbers which were hyperdiploid. The TEM-treated variants also had a larger proportion of cells which were triploid or hypotetraploid. This would explain the incomplete specificity of the variants isolated after treatment with TEM.

While it appears possible to use heterozygous tumours for isolating genetical variants specifically compatible with one of the parental strains, the actual nature of the mechanism of variant production remains dubious.

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