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Is micronucleus induction by aneugens an early event leading to mutagenesis?

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This study was designed to investigate a previously unidentified potential mechanism for mutation induction as well as to clarify a biological consequence of micronucleus formation. We compared the induction of micronuclei with mutation induction as measured by trifluorothymidine (TFT) resistance in mouse L5178Y cells using four aneugens: colcemid, diethylstilbestrol, griseofulvin and vinblastine. All four compounds induced micronuclei which appeared in the first cell cycle after treatment. More than 85% of the micronuclei induced by each compound stained positive for the presence of kinetochores implying that the micronuclei contained whole chromosomes. However, these same compounds were unable to induce TFT resistance under three different treatment regimes. We concluded that these compounds, under conditions where they induce primarily kinetochore positive micronuclei, were not able to induce mutations. Thus, the induction of micronuclei containing whole chromosomes harboring a selectable gene is not an early event leading to mutations in these cells.

Introduction

A number of investigators have monitored trifluorothymidine (TFT) resistance in L5178Y mouse cells to identify the mutagenic activity of chemicals (Clive *et al.*, 1972; Amacher *et al.*, 1980; Oberly *et al.*, 1984; Caspary and Myhr, 1986; McGregor *et al.*, 1989, 1991; Myhr *et al.*, 1990; Stopper *et al.*, 1993b). The L5178Y mouse cell line, clone 3.7.2c, is heterozygous at the thymidine kinase (*tk*) locus. The inactive *tk* allele possesses a point mutation at position 489 changing the code from a cysteine to a glycine (Liechty *et al.*, 1993). Both chemical and physical agents can induce TFT resistance in this cell line by producing the homozygote in which both *tk* alleles are inactivated. Cells heterozygous at selectable genes have a greater sensitivity to the mutagenic activity of chemicals than those cells that have the selectable gene on a single copy chromosome (Clive, 1985; Evans *et al.*, 1986; Moore *et al.*, 1989; McGregor *et al.*, 1994). Presumably the difference in sensitivity is due to the survival of a greater spectrum of mutant types in cells that are heterozygous. It is believed that mutants containing multi-locus lesions, such as major deletions and whole chromosome losses, in the chromosome with the active *tk* allele survive because the lost essential genes that flank this allele are present and active on the homologous chromosome which harbors the inactive *tk* allele (Evans *et al.*, 1986).

L5178Y mouse mutant colonies demonstrate a bimodal distribution of colony sizes and the colonies have been designated as small or large. The size of mutant colonies depends partially on the growth rates of the mutants during the cloning phase of the assay—slow growth leads to small colonies (Moore *et al.*, 1985). Large colonies are due to mutations within the active allele. Small colonies result in part from lesions that affect not only the active *tk* allele but a flanking gene whose expression modulates the growth rate of the cells. Cytogenetic studies have shown that 40–93% of the small colonies (Hozier *et al.*, 1985; Blazak *et al.*, 1986a,b,1989) exhibit translocations in which chromosomal material is attached to a break point in the distal end of chromosome 11 (chr 11) at a site close to the location of the *tk* allele (Hozier *et al.*, 1989, 1991). Molecular studies confirm the loss of large segments of the DNA in the region of the *tk* allele (Yandell *et al.*, 1986; Clive *et al.*, 1990; Liechty *et al.*, 1993). Thus, mutations can be formed when the *tk* allele is distal to, or located at the site of the break point at which the translocation is observed. However, not all small colonies are caused by the induction of large deletions or translocations of genomic material in the vicinity of the *tk* allele (Blazak *et al.*, 1989; Clive *et al.*, 1991).

The L5178Y mouse cell line detects many types of mutagenic lesions including those leading to small colonies. We have reported an *in situ* protocol that accurately detected these different lesions by capturing a greater proportion of the smaller colonies than do conventional protocols (Rudd *et al.*, 1990; Spencer and Caspary, 1994; Spencer *et al.*, 1994). In this procedure, cells were immobilized immediately after treatment and the proportion of slowly growing mutants to the total population remained constant during the cloning phase of the assay. This procedure led to a mutation rate that was 50-fold greater than that found with the conventional protocol because slowly growing mutants were not diluted out and each mutation led to one mutant colony (Rudd *et al.*, 1990). Because the number of mutations is measured, the mutation fraction (not the mutant fraction) is the parameter calculated as the measure of the mutagenic activity of a chemical (Rudd *et al.*, 1990; Spencer and Caspary, 1994; Spencer *et al.*, 1994). Because each chromosome would be expected to harbor some essential genes, whole chromosome losses might be expected to reduce the growth rate and produce small colonies. Therefore, we used this protocol to provide the greatest chance of detecting mutants containing whole chromosome losses. Such losses might be expected when aneugens induce micronuclei containing whole chromosomes. We have also established that L5178Y mouse cells are suitable for measuring micronucleus induction by chemicals (Stopper *et al.*, 1993a,b). Thus, assays for both mutations and micronuclei were performed in one cell system.

Micronuclei contain chromatin material within a membrane and are found in the cytoplasm of cells. Some micronuclei contain whole chromosomes; others contain chromosomal fragments. The two types of micronuclei can be distinguished from one another by anti-kinetochore-antibody-staining.

The fate of micronuclei as well as that of the cell harboring the micronucleus is unknown. Theoretically, a cell which has

completed mitosis and formed a micronucleus which contains whole chromosome or a chromosomal fragment harboring a selectable gene should be able to form a resistant colony if the genetic information in a micronucleus is not available for the cell. The homologous chromosome remaining in the nucleus might allow it to survive and divide. In support of this, it has been shown that L5178Y cells which are monosomic for chr 11 containing the tk^+ allele have been isolated and have survived (Evans *et al.*, 1986).

Micronucleus formation is a phenomenon whose biological consequences are unknown. It is a widely used toxicological endpoint *in vivo* (mouse bone marrow assay; Gudi *et al.*, 1992), *in vitro* (short term tests in culture cells; Fritzenschaf *et al.*, 1993; Matsuoka *et al.*, 1993) and in the biomonitoring of human populations (Fenech, 1993). Micronucleus formation has also been invoked as a possible mechanism for the loss of tumor suppressor genes, thus contributing to neoplastic cell transformation (Schiffmann and DeBoni, 1991).

The purpose of this investigation is twofold: (i) to investigate one potential biological consequence of the presence of micronuclei in cells and (ii) to determine if mutation assays can detect loss of chromosomes mediated by the induction of micronuclei containing whole chromosomes. To acomplish this, we determined optimal conditions for micronucleus induction and then used the same conditions to try to induce mutations in these cells.

Materials and methods

Chemicals

Bisbenzimide 33258, DES, griseofulvin, vinblastine, colcemid, ethyl methylsulfonate (EMS), hypoxanthine, methotrexate, thymidine, glycine, TFT, Tween 20 and FITC-conjugated goat anti-human antibody were purchased from Sigma Chemie GmbH (Deisenhofen, Germany). Dimethyl sulfoxide (DMSO) and formamide were acquired from Aldrich (Steinheim, Germany). CREST-serum (anti-kinetochore-antibody) was purchased from Biermann GmbH (Bad Nanheim, Germany).

Cell culture

Mouse L5178Y cells, clone 3.7.2c (Clive *et al.*, 1972), were cultured in suspension in RPMI-1640 medium supplemented with 95 U/ml penicillin, 95 μg/ml streptomycin, 0.25 mg/ml L-glutamine, 107 μg/ml sodium pyruvate (Sigma and Gibco), 0.05% pluronic (BASF Corp.) and 10% heat inactivated horse serum (Sigma and Hazleton Biologics). Cell cultures were grown in a humidified atmosphere with 5% CO_2 in air at 37°C.

In vitro micronucleus assay and kinetochore analysis

Expontentially growing L5178Y mouse cells with a doubling time of 10 h were treated for 4 h. The vehicle control was 1% DMSO or ethanol. After removing the chemicals by centrifugation and medium replacement, the cells were incubated for 5 h. The cells were then brought onto glass slides by cytospin centrifugation. Fixation was performed with methanol (−20°C, 1 h). For staining of nuclei and micronuclei, the slides were washed with distilled water, incubated with bisbenzimide 33258 (5 μg/ml, 3 min), washed with distilled water, and mounted for microscopy. Kinetochore staining was achieved by incubating the fixed cell preparations [after washing for 5 min in phosphate buffered saline (PBS)/0.1% Tween 20] with CREST serum for 75 min in a humidified chamber at 37°C. After washing twice for 5 min in PBS/0.5% Tween 20, the cells were incubated for 30 min with FITC-conjugated goat-anti-human antibody which had been diluted 1:100 in PBS/0.5% Tween 20, pH = 7.4. They were then washed twice in PBS/0.1% Tween 20 and counterstained with bisbenzimide 33258 (5 μg/ml, 3 min). This staining method provides for the simple scoring of nuclei and micronuclei but does not permit the visualization of individual cells. Thus, using a magnification of 1250×, we counted the number of nuclei in a field and then the number of micronuclei in that same field. Each data point represents the mean of three slides with 2000 nuclei evaluated per slide. All experiments were repeated to insure replication of results.

Mutation experiments

Cultures of mouse L5178Y cells were treated with methotrexate before each experiment to kill pre-existing TFT^r cells. To accomplish this, cells were incubated for 24 h in culture medium plus methotrexate (0.3 μg/ml), thymidine (9 μg/ml), hypoxanthine (15 μg/ml) and glycine (22.5 μg/ml). The cells were then incubated for at least 48 h in the same medium without methotrexate. To measure chemically induced mutations using the *in situ* procedure (Rudd *et al.*, 1990; Spencer and Caspary, 1994; Spencer *et al.*, 1994), cultures containing $1-10 \times 10^6$ cells in 5–10 ml medium were treated with DMSO (vehicle control) or chemicals (dissolved in DMSO). We treated the cells with the chemicals for 4 h, then washed the cells twice with fresh medium. In some experiments (Table IV) a recovery period of 5 h allowing the cells to grow in suspension was included. After that, from each tested culture, $0.5-5 \times 10^6$ cells were added to 50 ml of semi-solid culture medium (containing 0.25% granulated agar, Baltimore Biological Laboratories), plated into two plastic 100 mm culture dishes and allowed to solidify at room temperature. TFT^r cells were selected by adding an overlay of TFT to a final concentration of 8 μg/ml in 10 ml semi-solid medium after an expression time of 17–44 h (as specified in the Results and table legends). The cloning efficiency was determined by adding 600 cells to 100 ml of semi-solid medium and pouring into three 100 mm culture dishes. All plates were incubated for a total of 11–12 days at 37°C in 5% CO_2 for colony growth and the number of TFT^r colonies was counted.

Results

We treated L5178Y cells for 4 h with several doses of each of the four aneugens—colcemid, DES, griseofulvin and vinblastine—and determined whether the induced micronuclei (Figure 1) contained whole chromosomes (Table I).

We measured the micronucleus fraction 5 h after withdrawing the chemical from the medium. This time point was found to be optimal for these substances in preliminary experiments. The fact that the micronuclei were observed so early suggested that they were induced during the first mitosis after treatment, presumably by interference with the mitotic apparatus.

The highest doses examined were determined as follows. For colcemid and vinblastine, the highest doses accepted for evaluation were those that did not induce a large percentage of cells containing multiple micronuclei. At 0.5 μg/ml, colcemid induced 181 ± 12 micronuclei per 2000 cells and at 0.05 μg/ml, vinblastine induced 234 ± 82 micronuclei per 2000 cells. In both cases more than half of the micronuclei were in cells containing two or more micronuclei and, consequently, these concentrations were not included in Figure 1. Griseofulvin and DES were investigated only at soluble concentrations. Griseofulvin reached its detectable solubility limit at 200 μg/ml and DES at ~25 μg/ml in culture medium.

Colcemid, griseofulvin and vinblastine elicited an increased response with increasing dose up to the maximum concentration examined. DES, however, reached a maximum micronucleus fraction at ~12.5 μg/ml after which it decreased. It is possible that the solubility limit was reached at lower concentrations than those at which it was visibly detectable in the medium, which may have resulted in the decrease in the micronucleus fraction.

Table I. Presence of kinetochores in micronuclei

Substance	Dose (μg/ml)	Micronuclei/ 2000 cells	Percentage of micronuclei containing kinetochores
Control	1% DMSO	5	19.5 ± 4.9
Colcemid	0.1	69	94.7 ± 1.5
DES	12.5	35	89.3 ± 3.2
Griseofulvin	100	28	87.4 ± 2.8
Vinblastine	0.025	53	96.3 ± 2.1

Kinetochore staining was achieved by incubating the fixed cell preparations with CREST serum. For the percentage of micronuclei containing kinetochores, each data point represents the mean of three slides ± the standard deviation. For each substance, 100 micronuclei on each of three slides were scored for the presence of a signal.

With all four compounds, the lowest doses at which micronuclei were observed were relatively non-toxic based on a measurement of cloning efficiency (Tables II–IV).

Staining of kinetochores showed that all four compounds induced high percentages (>85%) of kinetochore-positive micronuclei (Table I). We concluded that the compounds

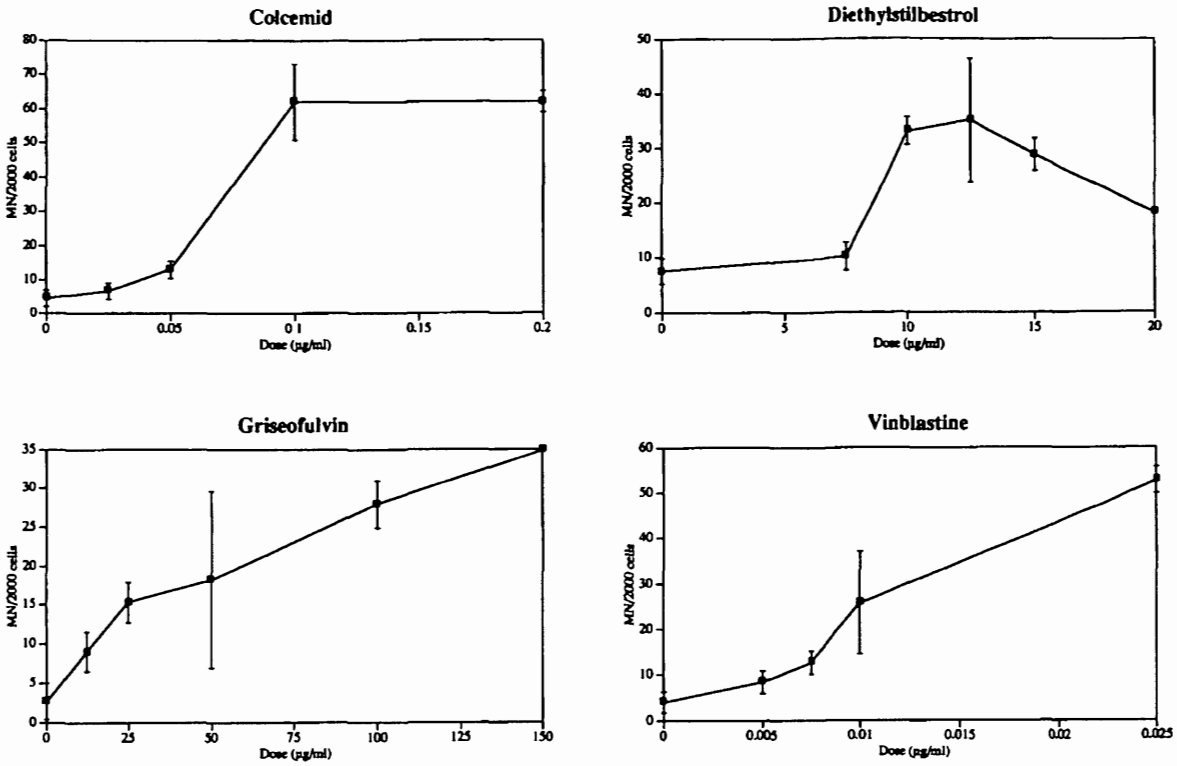

Fig. 1. Micronucleus induction by four aneugens. Mouse L5178Y cells were treated with the four chemicals at the concentrations shown. Standard deviations are shown by the bars on the graphs.

Table II. Absence of mutation induction in L5178Y mouse cells by aneugens—40–44 h expression

Substance	μg/ml	Experiment A			Experiment B			Experiment C		
		MnF	(RMnF)	CE	MnF	(RMnF)	CE	MnF	(RMnF)	CE
Colcemid	0	159 ± 5	(1.0)	0.66	118	(1.0)	0.68	276	(1.0)	0.62
	0.1	239 ± 40	(1.5)	0.54						
	0.2	239 ± 12	(1.5)	0.62	171 ± 35	(1.4)	0.51	346	(1.3)	0.70
	0.4	198 ± 63	(1.3)	0.55						
EMS	250	1106 ± 133	(7.0)	0.53	1054	(8.9)	0.59			
DES	0	272 ± 79	(1.0)	0.96	118	(1.0)	0.68	415 ± 83	(1.0)	0.37
	12.5	242 ± 52	(0.9)	0.98	172 ± 18	(1.5)	0.58			
	15							563 ± 21	(1.4)	0.21
	25	261 ± 1	(1.0)	1.01						
	50							347 ± 83	(0.8)	0.13
EMS	250	1683 ± 189	(6.2)	0.84	1054	(8.9)	0.59			
	300							3753 ± 561	(9.0)	0.22
Griseofulvin	0	288	(1.0)	0.50	118	(1.0)	0.68	276	(1.0)	0.62
	25	294	(1.0)	0.53						
	50	355	(1.2)	0.31						
	100	290	(1.0)	0.51						
	150	206	(0.7)	0.36	168	(1.4)	0.59	362	(1.3)	0.47
EMS	250	1298	(4.5)	0.59	1054	(8.9)	0.59			
Vinblastine	0	159 ± 5	(1.0)	0.66	276	(1.0)	0.62	220	(1.0)	0.71
	0.025	242 ± 28	(1.5)	0.39						
	0.05	207 ± 50	(1.3)	0.41	367	(1.3)	0.57	293	(1.3)	0.71
	0.1	172 ± 66	(1.1)	0.56						
EMS	250	1106 ± 133	(7.0)	0.53				1452	(6.6)	0.51

Mutations were measured using the *in situ* procedure. Treatment time was 4 h, expression time 40–44 h. MnF = mutation fraction. MnFs with '±' were performed in duplicate and the standard deviation is shown. MnFs without '±' were not conducted in duplicate. RMnF = relative mutation fraction. CE = cloning efficiency. DES = diethylstilbestrol. EMS = ethyl methanesulfonate.

Table III. Absence of mutation induction in L5178Y mouse cells by aneugens—17 h expression

Substance	μg/ml	Experiment A			Experiment B			Experiment C		
		MnF	(RMnF)	CE	MnF	(RMnF)	CE	MnF	(RMnF)	CE
Colcemid	0	46	(1.0)	0.66						
	0.2	29	(0.6)	0.56						
DES	0	23 ± 7	(1.0)	0.96	12 ± 1	(1.0)	1.12	32 ± 1[a]	(1.0)	0.96
	12.5	28 ± 1	(1.2)	0.98	26 ± 4	(2.1)	0.66	43 ± 5[a]	(1.3)	0.98
	20				20 ± 2	(1.7)	0.67			
	25	26 ± 13	(1.1)	1.01				38 ± 1[a]	(1.2)	1.01
EMS	250				15 ± 3	(1.3)	0.79			
	300	23	(1.0)	0.84				66 ± 21[a]	(2.1)	0.84
Griseofulvin	0	46	(1.0)	0.66						
	150	34	(0.7)	0.65						
Vinblastine	0	46	(1.0)	0.66						
	0.05	35	(0.8)	0.84						

Mutations were measured using the *in situ* procedure. Treatment time was 4 h, expression time 17 h ([a]22.25 h). MnF = mutation fraction. MnFs with '±' were performed in duplicate and the standard deviation is shown. MnFs without '±' were not conducted in duplicate. RMnF = relative mutation fraction. CE = cloning efficiency. EMS = ethyl methanesulfonate. DES = diethylstilbestrol.

Table III. Absence of mutation induction in L5178Y mouse cells by aneugens—5 h recovery + 40 h expression

Substance	μg/ml	Experiment A			Experiment B			Experiment C		
		MnF	(RMnF)	CE	MnF	(RMnF)	CE	MnF	(RMnF)	CE
Colcemid	0	344	(1.0)	0.62	441	(1.0)	0.56	361	(1.0)	0.53
	0.2	422	(1.2)	0.41	287	(0.7)	0.38	329	(0.9)	0.42
EMS	250	1115	(3.2)	0.67	941	(2.1)	0.70	894	(2.5)	0.79
DES	0	235	(1.0)	0.87						
	12.5	223 ± 32[a]	(0.9)	0.65						
	20	184 ± 40	(0.8)	0.57						
EMS	250	986 ± 23	(4.2)	0.85						
Griseofulvin	0	344	(1.0)	0.62	441	(1.0)	0.56	361	(1.0)	0.53
	150	341	(1.0)	0.37	226	(0.5)	0.69	352	(1.0)	0.58
EMS	250	1115	(3.2)	0.67	941	(2.1)	0.70	894	(2.5)	0.79
Vinblastine	0	344	(1.0)	0.62	441	(1.0)	0.56	361	(1.0)	0.53
	0.05	380	(1.1)	0.66	202	(0.5)	0.66	223	(0.6)	0.71
EMS	250	1115	(3.2)	0.67	941	(2.1)	0.70	894	(2.5)	0.79

Mutations were measured using the *in situ* procedure. Treatment time was 4 h, then cells were allowed to grow in suspension for 5 h before being plated for an expression time of 40–44 h. MnF = mutation fraction. MnFs with '±' were performed in duplicate and the standard deviation is shown. MnFs without '±' were not conducted in duplicate. ([a]Average of five treated cultures.) RMnF = relative mutation fraction. CE = cloning efficiency. EMS = ethyl methanesulfonate. DES = diethylstilbestrol.

examined induced the enclosure of whole chromosomes into micronuclei in these cells. In control cultures, which averaged ~3 or 4 micronuclei per 2000 cells, 19.5% of the micronuclei were kinetochore positive showing that micronuclei can contain chromosomal fragments in these cells. These micronuclei data are consistent with mechanisms in which mitosis is perturbed resulting in lagging chromosomes and the formation of micronuclei containing whole chromosomes.

Next, we examined the four aneugenic compounds and EMS (as a positive control) for mutagenicity as measured by the induction of TFT resistance in these cells. Three different modifications of the protocol were used.

A dose–response experiment for mutation was performed with each substance (Table II). Earlier investigations showed that the *in situ* expression of chemically induced mutation in L5178Y cells can be expected at ~40–50 h (Spencer and Caspary, 1994; Spencer *et al.*, 1994). Therefore, we chose a 40–44 h expression time and treated the cells using the same dose range as in the micronucleus experiments. EMS clearly caused an elevated mutation fraction in each of the experiments. The highest relative mutation fraction (RMnF) of 1.5 for the aneugens was not reproducible. We concluded that no significant increase could be observed with the four aneugens.

However, the optimal time for mutation would be expected to depend on the mechanism leading to mutation. It is believed that aneugens induce micronuclei by causing the chromosomes to lag during mitosis and this would occur during the first cell division after treatment. It would be expected that mutation expression would be earlier for treatment with aneugens than with treatment with compounds that form DNA adducts. Such compounds, for example EMS, can require up to two cell divisions for the mutation to be fixed. Therefore, we looked at the mutagenic response after a 17 h expression time. We observed no significant increase in the mutation fraction (RMnF ≤ 1.7) with any of the aneugenic substances at this early expression time (Table III), with the exception perhaps of DES at 12.5 μg/ml (experiment B). However this response was not duplicated in the other experiments shown or in other replicate experiments that we performed. As expected, the mutation fractions for EMS at a 17 h expression time were in the same range as for the aneugens.

Since aneugens presumbly act on the mitotic apparatus of cells

during mitosis, L5178Y mouse cells might be especially susceptible to dying if they were in mitosis at the time of plating. To counter that possibility, we modified the mutation protocol to allow a 5 h recovery period between treatment and plating of the cells and assessed the mutagenic response after an additional expression of 42 h. Again we observed no response for the four aneugens (Table IV).

Under the various conditions used here, none of the aneugens—colcemid, DES, griseofulvin and vinblastine—induced TFT resistance with any of the treatment protocols used, at doses in the same range as those in which they produced micronuclei. EMS showed a significant induction of mutation fractions in our experiments.

Discussion

Under conditions where they induced micronuclei, the aneugens examined did not induce mutations in L5178Y mouse cells at the *tk* locus. This suggested that the induction of micronuclei containing whole chromosomes was not an early event leading to phenotypically expressed mutations in these cells. However, if chromosomes were not randomly excluded from the nucleus to form a micronucleus, it is possible that chromosome 11 might not be found in any of the micronuclei. If this were the case, the L5178Y mutation assay would not be an appropriate assay to measure the results of micronucleated cells because it measures mutation at chromosome 11. However, it should be noted that there is no evidence suggesting that chromosome 11 is not being expelled from the nucleus to form a micronucleus.

If micronucleated cells survive and grow, their effect on the mutation fraction should be readily observed. In our experiments, we examined aneugen doses that induced ~40 or more micronuclei per 2000 cells. There are 40 chromosomes in the mouse; if we assume that whole chromosomes are randomly excluded from the nucleus, then the number of cells which form micronuclei containing chr 11 containing the tk^+ allele is one per 2000 cells. If each of these cells were to survive to form a mutant we could expect an induced mutation fraction of 500 mutations per million cells. The spontaneous mutation fractions in our experiments generally were in the range of 100–300 mutations per million cells at 40–44 h expression and <50 mutations per million cells at 17 h. Thus, if the micronucleated cells survive and divide, the additional induced mutation fraction of 500 mutations per million cells should be detectable. If micronucleated cells did not survive, there would be no mutants to count because dead mutants cannot be scored.

The reason for the seeming refractivity of L5178Y cells to the mutagenic activity of aneugens may have to do with the fate of the micronucleus, which is not known. A micronucleated cell might die, resulting in the disintegration of the micronucleus, or it might be cytostatic and not divide further to form a mutant colony. Alternatively, a micronucleus might disintegrate resulting in a cell lacking a chromosome and the cell in turn might die in which case it would not result in a viable mutant. It could survive with a reduced complement of chromosomes or duplicate the remaining chromosome and possess a chromosome complement containing two identical chrs 11 each containing the inactive *tk* allele. L5178Y cells that lost a whole chr 11 containing the inactive *tk* allele have been described and shown to grow in culture (Evans *et al.*, 1986). This suggests that L5178Y cells lacking a chr 11 can survive. Alternatively, the chromosome may reintegrate into the nucleus of the cell, reincarnating a wild-type cell that does not possess a mutant phenotype.

It appears that L5178Y mouse cells cannot be expected to detect aneugens at the *tk* locus. It is not known whether this conclusion can be generalized to other mutation assay systems. The four aneugens that we chose, colcemid, DES, griseofulvin and vinblastine, have not been studied extensively for their mutagenic properties in mammalian cells. We could find information only on DES. The evidence presented for DES indicates that this compound is not mutagenic in mammalian cells in culture (Barrett *et al.*, 1981, 1983; Drevon *et al.*, 1981; Mitchell *et al.*, 1988; Myhr *et al.*, 1988). However, some variability has been reported (Clive *et al.*, 1979; Myhr *et al.*, 1985). However, the positive responses in these latter studies occurred at doses that may have been insoluble (≥25 μg/ml), though other effects may be responsible for the responses observed. The *hprt* locus would not be expected to detect aneugenic compounds because of the unavailability of a homologous chromosome. It appears that if an aneugen is to be detected, another assay like the micronucleus test needs to be used.

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