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THE APPLICATION OF MAMMALIAN CYTOGENETICS TO MUTAGENICITY STUDIES

J. G. Brewen

Biology Division, Oak Ridge National Laboratory,* Oak Ridge, Tennessee 37830

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*Operated by the Union Carbide Corporation for the Energy Research and Development Administration.

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Introduction

The technical advances in mammalian cytogenetics over the past two decades have advanced this subdiscipline of genetics from a highly descriptive science to one with a high degree of objective quantitation. Concomitant with the technical advances, there have been increasingly clear demonstrations that many of the genetic end points measured in mammalian mutagenesis studies are either associated with, or the result of, structural chromosome aberrations. Thus the rationale can be used that the direct study of chromosome damage can be applied to the estimation of heritable genetic defects. Such estimations have been made for the recovery of reciprocal translocations in offspring of irradiated parents based on cytogenetic data derived from peripheral leukocytes and maturing germ cells.[1]

There are many cytogenetic techniques available to the research scientist, most of which are unique for the particular end point the investigator wishes to measure. Rather than catalogue the techniques and their usefulness, it is probably more instructive to critically evaluate two specific problems and to illustrate how various cytogenetic procedures have been used to study these problems. For the purpose of diversity I have chosen a problem involving a chemical mutagen and a problem involving ionizing radiation. These are, specifically, the production of dominant lethals and translocations in male mice by the trifunctional alkylating agent triethylene melamine and the induction of mutations, including chromosome aberrations, in maturing mouse oocytes by low linear energy transfer radiations.

Induction of chromosome damage in germ cells by TEM:

Triethylene melamine (TEM) is a trifunctional alkylating agent that has been shown to produce reciprocal translocations and dominant lethality in primary spermatocytes[2] and post-meiotic male germ cells[3] of the mouse. It has also been shown to be an efficient inducer of dominant lethality in the later stages of maturing mouse oocytes.[4-7] Treatment of differentiating spermatogonia or spermatogonial stem cells, however, results in the recovery of very few, if any, mutagenic events in the subsequent primary spermatocytes or F_1 of treated males.[8]

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These results are in accord with those obtained with most alkylating agents.[5-7,9-11] It has been suggested[12] that the primary reason for the results obtained with spermatogonial cells is that alkylating agents have a time-specificity of action that coincides with replicative DNA synthesis, as shown by Evans and Scott for maleic hydrazide[13] and nitrogen mustard.[14] The inability to recover genetic effects from S-phase-treated spermatogonia is most likely due to the intrinsic sensitivity of these cells to killing by TEM, coupled with the loss of the majority of viable balanced chromatid interchanges through mitotic segregation.

The effects on oocytes and postmeiotic male germ cells can be attributed to the lack of repair of premutational lesions for at least one of three reasons. These reasons are: (1) the particular cell type does not possess repair capability, as demonstrated by Sega,[15,16] for mid-spermatids and spermatozoa; (2) there is not sufficient time for repair, as is probably the case for late maturation stages of oocytes; and (3) some compounds produce lesions that the cells are incapable of repairing with high efficiency. The retention of these lesions in the genome of the germ cell could then lead to chromosomal damage at the time of pronuclear DNA synthesis in the 2N zygote. This certainly seems to be the case when spermatids, spermatozoa, and oocytes are treated with methyl methanesulfonate.[17,18]

Recently Generoso et al.[2] proposed that the chromosome aberrations induced by treating pachytene primary spermatocytes with TEM are formed independently of DNA synthesis. They postulated this in spite of the fact that analysis of diplotene chromosomes after pachytene treatment resulted in a negligible yield of aberrations. They state: "First, an intervening round of DNA synthesis does not appear to be necessarily a prerequisite for the formation of exchanges," based on the fact that X-irradiation of pachytene resulted in chromosome aberrations observed at diplotene. This observation is not surprising, nor is the absence of TEM-induced aberrations in parallel experiments, if replicative DNA synthesis is an integral part of the mechanism of chemically induced chromosome damage.

We have recently studied the production of chromosome aberrations in various cell types by TEM. These studies included experiments with peripheral human leukocytes, pre-leptotene through pachytene mouse primary spermatocytes, and maturing mouse oocytes. With the exception of the experiments on mouse oocytes, the treatment with TEM was always coupled with exposure to [^{3}H]dThd in order to identify cells in the S phase.

Tables 1–3 summarize the data obtained in these experiments. The data from the leukocyte studies show three things quite clearly (Table 1). First, there is a definite cell-stage specificity for the induction of chromosomal aberrations, with late-G_1 and S being the most sensitive. Second, the great majority of the aberrations induced in all stages of the cell cycle, including G_1, are chromatid-type. Third, there is a significant increase in chromosome-type aberrations (dicentrics) resulting from G_0 (-2 to 0 h) and G_1 treatment.

TABLE 1

Frequencies of chromosomal aberrations observed in human leukocytes after treating different stages of the cell cycle with 1×10^{-5} TEM

Cell stage treated	No. of cells scored	Chromatid deletions (%)	Chromatid exchanges (%)	Iso deletions (%)	Rings plus dicentrics (%)	Achromatic lesions (%)
G_0	300	4.0	0.7	11.0	3.3	5.0
Early G_1	300	11.3	6.3	22.0	5.3	8.0
Late G_1**	300	37.3	38.0	61.0	5.3	19.7
G_1 control	300	2.7	0.0	0.7	0.3	1.3
S*	300	18.7	7.3	37.7	0.0	19.0
S control	300	2.7	0.0	3.7	0.0	0.3
G_2**	300	8.0	0.0	2.7	0.3	3.3

* Labeled cells only.

** Unlabeled cells only.

The data obtained from treating oocytes and analyzing them at either metaphase I or at the first zygotic cleavage division[19] (Table 2) show that an intervening round of replicative DNA synthesis (pronuclear) results in many more chromatid aberrations.[20] Analysis of metaphase I showed a small, but significan increase in aberrations.[20]

TABLE 2

The frequency of chromatid aberrations observed in metaphase I oocytes and the female pronuclear chromosomes following treatment of female mice with either 0.8 or 1.6 mg/kg TEM

Dose (mg/kg)	Interval (days)	Metaphase I			First cleavage zygotes			
		No. of cells scored	Deletions (% ± SE)	Exchanges (% ± SE)	No. of cells scored	Deletions* (% ± SE)	Exchanges* (% ± SE)	Cells with multiples** (%)
0	0.5	200	0.5 ± 0.5	0.0	50	0.0	0.0	0.0
0	4.5	—	—	—	50	2.0 ± 2.0	0.0	0.0
0.8	0.5	150	0.7 ± 0.7	0.0	75	47.9 ± 8.1	26.8 ± 6.1	5.3
0.8	2.5	125	0.0	0.0	50	16.0 ± 5.7	4.0 ± 2.8	2.0
0.8	4.5	100	3.0 ± 1.7	0.0	50	4.0 ± 2.8	2.0 ± 2.0	0.0
1.6	0.5	150	2.0 ± 1.2	0.7 ± 0.7	75	193.1 ± 25.8	105.4 ± 16.9	61.3
1.6	2.5	145	1.4 ± 1.0	0.0	50	44.9 ± 9.6	16.0 ± 5.7	2.0
1.6	4.5	110	2.7 ± 1.6	1.8 ± 1.3	90	11.1 ± 3.5	1.1 ± 1.1	2.2
1.6	6.5	125	1.6 ± 1.1	0.0	50	8.2 ± 4.1	12.2 ± 5.0	4.0
1.6	10.5	125	0.8 ± 0.8	0.0	75	13.5 ± 4.2	1.4 ± 1.4	1.4

* These frequencies are based on total number of cells less those with multiple aberrations that could not be quantitated.

** These represent cells that had too much damage to be quantitatively analyzed.

Treatment of primary spermatocytes (Table 3) results in a very cell-stage-specific response. Cytogenetic analysis of diplotene indicated that very few aberrations occurred in cells until the first group of labeled cells appeared. The low yield of aberrations from unlabeled pachytene stages agrees with the data of Generoso et al.,[2] and the high frequency observed in cells treated in premeiotic S confirms the expectation that the involvement of replicative DNA synthesis is required.

TABLE 3

Frequency of labeled diplotene-diakinesis figures and chromosome aberrations at various intervals following treatment of primary spermatocytes with 1.0 mg/kg TEM and [^{3}H]dThd (controls)

Time interval (days)	Treatment	No. of cells scored	Chromatid plus isochromatid deletions (%)	Chromatid exchanges (%)	Labeled figures (%)
6	[^{3}H]dThd	200	0.0	0.0	0.0
6	TEM	400	0.0	0.0	0.0
9	[^{3}H]dThd	200	0.0	0.0	0.0
9	TEM	400	0.25 ± 0.25	0.0	0.0
11	[^{3}H]dThd	100	0.0	0.0	0.0
11	TEM	200	0.5 ± 0.5	0.0	0.0
12	[^{3}H]dThd	100	0.5 ± 0.5	0.0	28.5
12	TEM	400	12.8 ± 1.8	0.5 ± 0.4	84.5
13	[^{3}H]dThd	100	0.5 ± 0.5	0.0	70.5
13	TEM	150	68.7 ± 6.8	0.0	95.7

All in all, the data show quite clearly that TEM is capable of producing a low level of aberrations without replicative DNA synthesis, but that it is much more effective as a clastogen when there is an intervening round of DNA synthesis. Thus, it is conceivable that some of the dominant lethal effects observed by Generoso et al.[2] resulted from prefertilization aberrations; however, the great majority probably resulted from aberrations formed during pronuclear DNA synthesis.

In studies where postmeiotic male germ cells were treated with TEM, or with Trenimon, three reports[21-23] show that chromatid- and chromosome-type aberrations were seen at later

(4–16 cell stages) cleavage stages. The chromosome-type aberrations could have been derived from chromatid-types that occurred at the first cleavage division. The data of Binkert and Schmid[22] strongly suggest this, in that karyotype analysis of several cells from 4- and 8-cell embryos shows that the chromosome-type aberrations were not present in all cells. This can mean that the embryo consisted of cells derived from the two segregation types expected from chromatid-type aberrations. Burki and Sheridan[21] have shown, however, that chromosome-type aberrations are obtained at first cleavage. These could have arisen from isochromatid–isochromatid exchanges.

Cytogenetic effects of low linear energy transfer radiation on mouse oocytes:

For the past several years a considerable dialogue has existed on the subject of the interpretation of the specific locus mutation data derived from irradiation of mouse oocytes. Russell[24-32] maintains that specific locus mutations arise as the result of a one-track process, and that the almost D^2 (dose squared) dose-response kinetics are a reflection of inhibition of repair of premutational lesions at higher doses and dose rates. Consequently, at low doses and dose rates, where repair would be minimally inhibited, the mutation rate is expected to be low; experimental data support this view. Abrahamson and Wolff,[33] however, argue that the mutations arise from small chromosome deletions that are produced predominantly by a two-track process. A complicating factor that must be considered in the interpretation of the data is the apparent variation in radiosensitivity of the various stages in oocyte maturation.

It has been shown that the primary oocyte (resting) is particularly insensitive to mutation induction.[27] There is also a very large variation in the sensitivity of the maturing oocytes to dominant lethal induction.[34,35] Just prior (< 8.5 h) to ovulation, the oocyte is very sensitive; this sensitivity drops rapidly with time until 3 days prior to ovulation, when it begins to increase again. A similar pattern has been observed when metaphase I oocytes have been analyzed for structural chromosome damage after acute X-ray doses.[36-38]

We have investigated these various problems in more detail, using chromosome damage in the metaphase I oocyte as the genetic end point. Three basic experiments were done with young (8–12 weeks of age) female CD1 mice purchased from Charles River Breeding Laboratories, Inc. The variation in oocyte sensitivity as a function of time between irradiation and ovulation was studied in detail with a 300-R X-ray exposure and in part with 50-, 100-, and 200-R exposures. The effect of chronic γ-ray exposures was analyzed in oocytes exposed during a period of presumed uniform sensitivity. Finally, fractionation experiments were conducted to determine if X-ray dose had any effect on the time required for repair of the lesions that interact to form chromatid exchanges. In all the studies performed, superovulated metaphase I

oocytes were isolated after colchicine arrest induced by intraperitoneal injection of colchicine 12–16 h before oocyte collection.

Figure 1 summarizes the data obtained on stage sensitivity. It is seen that the frequency of aberrations rises as a function of time after irradiation, reaching a peak 8–9 days post-exposure, and then remains relatively constant, but with some fluctuations. It is felt that these later fluctuations may be due to errors resulting from small sample sizes. Sterility, as adjudged by the failure to obtain oocytes after superovulation, occurred between 24 and 29 days after exposure at all doses.

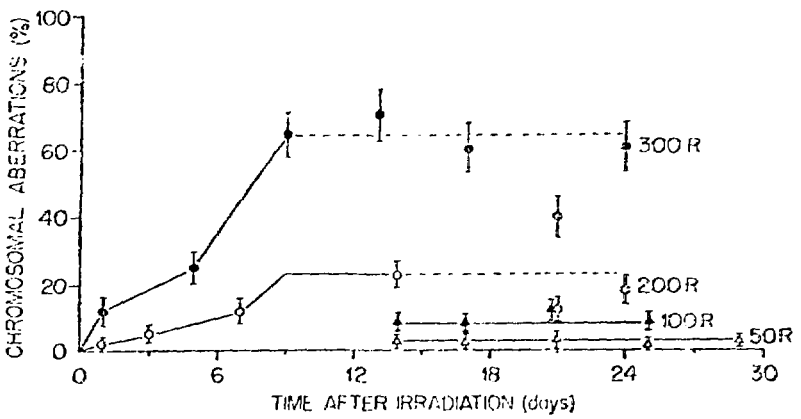

Fig. 1. The yield of total chromatid aberrations at various times following acute X-ray doses of 50 R (△), 100 R (▲), 200 R (○), and 300 R (●) delivered to mouse oocytes.

The present cytogenetic data on stage sensitivity agree with those previously published.[36-38] The apparent low sensitivity in the first week after irradiation may have some bearing on the observation by Russell that, in older females, the specific locus mutation frequency was significantly higher in the second litters as compared with the first. This is because most of the offspring in the first litters would be conceived in the first week after exposure, and the second litters would have been derived from earlier, more sensitive, maturation stages.

Figure 2 summarizes the cytogenetic data obtained after acute X-ray and chronic γ-ray exposure at 14 days and 8–16 days before ovulation, respectively. Following acute X irradiation, both chromatid interchanges and chromatid deletions increase with dose in a fashion indicating a very significant two-track process in their formation. In this sense the data agree, qualitatively, with published data on the induction of specific locus mutations. Chronic γ-ray exposure during the period of peak sensitivity of the maturing oocytes results in a low yield of chromatid aberrations.[39] In fact, the reduction in yield at 478 R, which is a dose of comparison, is intermediate to that seen for specific locus mutations at higher and lower dose rates. Thus chromatid aberration induction agrees, qualitatively, with specific locus mutation induction.[25]

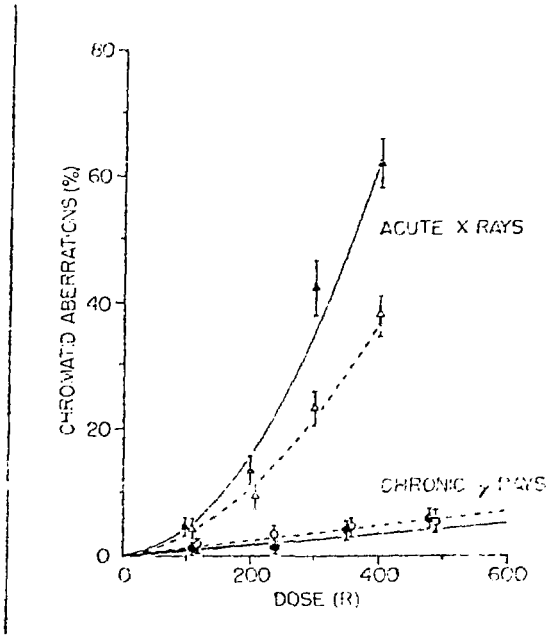

Fig. 2. The yield of chromatid aberrations (—, interchanges; ---, deletions) following either acute (△, ▲) or chronic (○, ●) irradiation of mouse oocytes during their period of peak sensitivity.

Table 4 summarizes the data from the fractionation experiments. For simplicity, only the chromatid interchange data are presented. These data show that the magnitude of the first dose does not alter the time necessary for repair to occur, thus implying that the repair system is not inhibited by radiation dose.[39] This conclusion is based on the fact that an additive yield ($Y = Y_{Di} + Y_{Dj}$) is obtained at the same fractionation interval regardless of the magnitude of the first dose. Had the magnitude of the first dose influenced repair, interaction (or the yield obtained by administering the entire dose at once) would have been expected at increasingly longer fractionation intervals. These data (as do the specific locus data of Russell[29] and Lyon[40]) also suggest that repair occurs rapidly and not over a period of weeks, as must be argued if it is assumed that DNA synthesis "fixes" the premutational lesion in the genome. This latter assertion is based on the facts that: (1) no DNA synthesis occurs in oocytes and (2) DNA synthesis has been shown by Kimball[41] to be important in radiation-induced mutation fixation.

The data on chromatid aberration induction in maturing oocytes suggest that structural aberrations are a reasonable qualitative measure of specific-locus and dominant-lethal effects. Since most specific locus mutations induced in oocytes have been identified as chromosome deletions,[42] it follows that the present data can be used as a model for evaluating the nature of the induction of specific locus mutations. Thus it appears that the dose-response kinetics of specific locus mutation induction in maturing oocytes can best be explained by the contribution

TABLE 4

Frequencies of observed and expected chromatid interchanges following various fractionation regimes of 400 R of acute X rays

Dose (R)	Fractionation interval (min)	No. of cells scored	Interchanges observed (% ± SE)	Expected interaction (%)	Expected additivity (%)
0	0	300	0.0	—	—
200	0	225	13.3 ± 2.4	—	—
400	0	225	55.6 ± 5.0	—	—
200 + 200	90	200	54.0 ± 5.2	55.6	26.6
200 + 200	135	200	43.5 ± 4.7	55.6	26.6
200 + 200	180	150	30.0 ± 5.4	55.6	26.6
0	0	200	0.0	—	—
100	0	265	4.2 ± 1.3	—	—
300	0	236	42.4 ± 4.2	—	—
100 + 300	90	237	68.4 ± 5.6	—	—
100 + 300	135	204	62.8 ± 5.6	68.4	46.6
100 + 300	180	280	52.9 ± 4.3	68.4	46.6
300 + 100	90	125	62.4 ± 7.1	68.4	46.6
300 + 100	135	239	61.5 ± 5.1	68.4	46.6
300 + 100	180	280	52.5 ± 4.3	68.4	46.6

of a large two-track component. Furthermore, since the dose-response kinetics best fit the model $Y = a + bD + cD^2$, it is reasonable to conclude that chronic exposure will result in a large reduction in yield as has been shown by specific locus tests. The magnitude of this reduction, however, will be influenced by the contribution of the two-track component (cD^2).

It appears that cytogenetic procedures are adequate to evaluate certain mutagenic end points. The precaution is added, however, that a thorough understanding of the genetic end points and concepts of their production is necessary before chromosome analysis can be utilized as a conclusive test. The issues discussed in the current article are far from settled, and considerable research is required before these seemingly basic problems are solved.

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