1976

DOSE-RESPONSE RELATIONSHIPS OF TRIETHYLENEMELAMINE ON VISIBLE CHROMOSOME ABERRATIONS IN MOUSE BONE MARROW

Submitted in Partial Fulfillment of the Requirements for Graduation with Honors to the Department of Biology at Carroll College, Helena, Montana.

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March 31, 1978

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ACKNOWLEDGEMENTS

We wish to thank our faculty advisor and readers, Dr. James J. Manion, Dr. John A. Christenson, and Mr. Guido M. Bugni for their time and advice. Dr. M. Manandhar and Dr. Benjamin A. Jackson from the Department of Teratology and Mutagenicity of Lederle Laboratories deserve special recognition for their donations of time, suggestions, guidance, and triethylenemelamine. We extend our gratitude to Dr. Jack H. Stimpfling, director of The McLaughlin Research Institute, Great Falls, Montana for his generous donation of animals. We also wish to thank Dr. Charles W. Watkins, Vancouver, Washington for securing of our research literature. We are also indebted to Mr. Robert Thennis for furnishing some of the experimental equipment. Finally, we wish to acknowledge the Department of Biology at Carroll College for the opportunity to conduct this thesis and for use of their facilities and equipment.

ABSTRACT

Cytogenetic analysis of mouse bone marrow chromosomes after treatment of the experimental animals with triethylenemelamine (TEM) revealed an overall increase in the number of chromosome aberrations. A dose-response curve for TEM is given and its significance discussed. An unexpected increase in the number of rings as well as a relatively constant value for the aberrations/cell ratio in each of three dosage groups (and control) were observed. Possible mechanisms are given attention.

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INTRODUCTION

All pharmacological and toxic effects are dose dependent — their activity increases as the dosage administered is increased (Frohberg, et al., 1973). The dosage response curves define characteristics of this activity. The curves permit determination of the range of dosages from which a predictable response can be deduced. The response to a particular dosage is important in determining the effective range so that the chemical can be used in therapy or in evaluation of similar pharmacologic or toxicologic substances and their activities.

TEM is very similar in structure and function of some anti-neoplastics (cancer treating agents) currently in use (i.e., ThioTEPA). TEM has been proven to be a mutagen in mammalian systems (Matter, et al., 1975; Soares, et al., 1977).

TEM is used as a positive control to test suspect mutagens (Soares, et al., 1977). The more we can know about any control, in this case, TEM, the more we will know about anti-neoplastics. One of the most efficient and effective ways to evaluate any drug is by its dose-response relationships. Dose-response relationships have been determined for TEM using micronuclei[1], dominant-lethal and spermatogonial cell studies.

The purpose of this experiment was to determine a dose-response relationship for TEM by examining the chromosomes of mouse bone marrow.

[1]Chromosome fragments lag behind in anaphase and form micronuclei in the cytoplasm, which are visible after the cells have expelled their nuclei (Matter, et al., 1974).

LITERATURE REVIEW

General Overview

Presently there is an increasing concern about possible mutagenic capabilities of a variety of drugs and chemical agents. These substances are thought to be hazardous to man by causing gene mutations as well as chromosome aberrations. The testing of animals under experimental controls is the most productive way to determine the possible consequences of chemical and drug use. Most testing is done on the chromosomes of mammals by examining the meiotic or mitotic metaphases (Matter, et al., 1974). This method is useful in determination of the potential mutagenic effects of various chemicals, but progress and scoring aberrations by physical count are slow.

Probably one of the most commonly used group of mutagens and anti-neoplastics is the alkylating agents, especially the "nitrogen mustard series" (Boesen, et al., 1969). Contained in this group is TEM which is a proven mutagen in mammalian systems (Soares, et al., 1977). It has been tested very extensively in rats and mice, and has been found to be a potent mutagen at dosages where it was of low toxicity to test animals (Soares, et al., 1977). Because of extensive testing TEM has been singled out as a positive control in experimentation designed to test suspect mutagens (Soares, et al., 1977).

The effects of mutagens can be examined by visible structural chromosome analysis. Two new ways of determining visible mutagen effects are by 1) micronuclei and 2) premature chromosome

condensation (PCC) (Matter, et al., 1975). It was found that TEM induced dominant lethal mutations, heritable translocations, and other chromosomal anomalies in various stages of the spermatogenic cycle of the mouse (Hitotsumachi, et al., 1976; Matter, et al., 1975). Dominant lethal studies with TEM showed a characteristic stage-specificity and dose-response relationship in post-spermatogonial stages of the mouse (Matter, et al., 1975).

Alkylating Agents

All alkylating agents are grouped together not because of their structural similarity, but because of the type of reactions they are all capable of undergoing —— the ability to add alkyl groups to water or to a wide range of functional groups (Boesen, et al., 1969).

Alkylating agents are attacked by nucleophilic sites on the nucleic acids. This adds a portion of alkylating agents to the nucleophilic sites. The reactions of alkylating agents with nucleic acids, especially those of DNA, were extensively examined experimentally during the 1960's and 1970's. Mutagens were postulated to change the DNA structure (Ludlum, 1972).

When DNA reacts with an alkylating agent the result is a phosphotriester. Originally extreme interest was placed on the substitution of guanine in the seven position, which change was to have accounted for 90% of the total alkylation. It was later demonstrated that the phosphotriester was the minor reaction product

(Strauss, 1975). It was theorized that the substituted seven-position guanine base might mispair with thymine or uracil in nucleic acid transcription or replication (Ludlum, 1972).

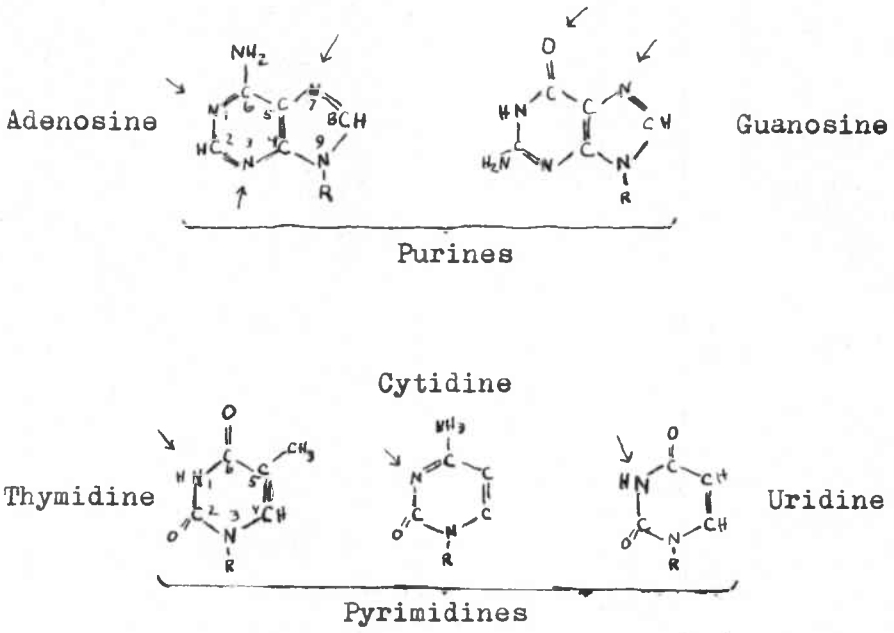

Figure 1. Reactive sites of alkylating agents in nucleosides. The reactivity of alkylating agents is the same in both RNA and DNA corresponding positions. The seven position of guanosine has been reported as the most reactive to alkylating agents, and they have been reported to react with DNA bases in a decreasing order of: guanine > adenine > cytosine (Ehling, et al., 1968; Ludlum, 1972; Strauss, 1975).

The mutagenic efficiency of an alkylating compound is in some way related to the number of active groups through which alkylation is possible (Fahmy, et al., 1955). It followed then that the monofunctional compounds are the weakest mutagens and that the polyimines (three active groups) were the most effective. Also, some alkylating agents were most effective in the induction of visible mutations (Fahmy, et al., 1955).

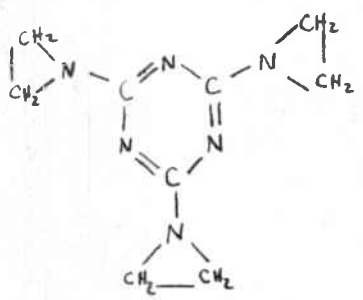

Figure 2. Chemical structure of triethylenemelamine. This compound is also identified by the IUPAC system as 2:4:6-tri(ethyleneimino)-1:3:5-triazine. The three three-membered rings are the active components of this alkylating agent.

Chemical Mechanisms of Alkylation

Initially what occurs in alkylation is the conversion of H-X to R-X. The source of the alkyl group (R) may be an alkylating agent from some cyclic compounds ($\overset{C-C}{Y}$). The biological reactions of interest are the ones involving the combining of the alkyl group to X through an oxygen, nitrogen, or sulfur atom (Price, 1958).

There are two recognized mechanisms for alkylation: 1) first order nucleophilic substitution (S_N1) and 2) second order nucleophilic substitution (S_N2). The S_N1 reaction proceeds in two steps. The first is the rate determining step of the reaction, which is also called the R-Y bond ionizing step. This step is usually aided by a polar solvent which helps to stabilize the resulting ions. The second step is extremely fast and is an indiscriminate reaction, with the resulting alkyl carbonium ion (R^+) from the first step reacting with a solvent molecule or a new anion (X^-). The concen-

tration of the (X^-) ion does not affect the rate of this reaction as the rate depends only on the concentrations of the alkylating agent and the solvent (Price, 1958). (Fig. 3)

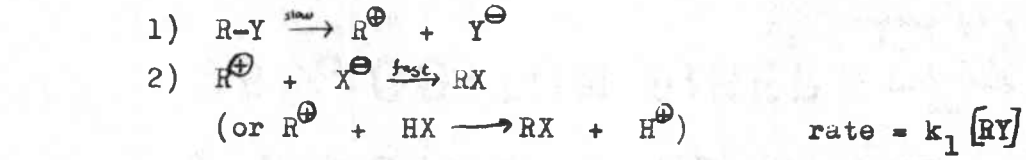

Figure 3. Representation of S_N1 reaction and its rate determining equation. R-Y, alkylating agent; X^-, compound being alkylated; RX, alkylated end product. The rate of this reaction is determined by the slow step, hence, it is proportional to the concentration of the alkylating agent.

In the S_N2 reaction, the "bond breaking and making" occur simultaneously. In this reaction the anion (X^-) has an important effect on the rate of the reaction, due to the two effects of its concentration and structure. (Fig. 4)

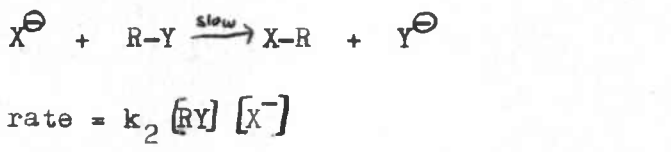

Figure 4. Representation of S_N2 reaction and its rate determining equation.

The ethyleneimines react by either S_N1 or S_N2, but the distinguishing feature about them is their reactive intermediate. The ring structure opens up to form a "linear" chain carbonium ion. (Fig. 5) This carbonium ion will react differently according to the pH (Ross, 1958).

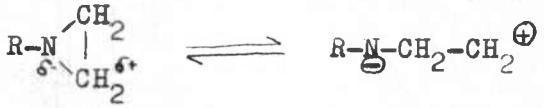

Figure 5. Reactive intermediate of ethyleneimines.

MATERIALS AND METHODS

Prior to the experiment, 43 male mice were weighed for 3 consecutive days. Their mean weights were used as the basis for the preparation of the TEM and colchicine solutions. Both solutions used distilled water as the solvent and were prepared immediately before injection.

The experiment consisted of 3 groups of 10, 12-18 week old, strain B10.BR male mice injected with TEM (Batch J-0710) each day for five days. Injections were given intraperitoneally (i.p.) in the following dosages: Group I, 0.15 mg TEM/kg of body weight; Group II, 0.40 mg/kg; and Group III, 0.70 mg/kg. Dilutions were made so each mouse was injected with 0.5 ml of solution. The control consisted of 10 untreated males.

On the sixth day, all groups (including control) were injected i.p. with 4.0 mg/kg colchicine. The mice were killed 2 hr later. The femoral bones were dissected out and the epiphyses cut off. The bone was held over a centrifuge tube and a 22 gauge hypodermic needle was inserted into the marrow cavity. Using a slight twisting action, the marrow was flushed out with a 1% sodium citrate solution at 37°C. The sample was filtered through sterilized gauze into another centrifuge tube and placed in a 37°C water bath for 8-10 min then centrifuged at 600 rpm for 5 min. After centrifugation, all the supernatant fluid was removed and 7 ml of freshly made fixative (1:3 mixture of glacial acetic acid : absolute methyl alcohol) was added without disturbing the cell mass.

After 16 hr of refrigeration, the first 3-5 ml of supernatant were discarded. The pellet of cells was broken up and washed three times with 3-5 ml of fixative. The resulting pellet was collected after all the fixative had been removed. The cells were resuspended in 0.4ml of fixative.

Three or four drops of cell suspension were placed on ethyl alcohol cleaned slides. This was momentarily ignited and the slides were allowed to dry for 1 hr.

The stock solution of stain was prepared by Wolbach's Giemsa Method (Luna, 1960). The working stain was prepared by diluting the stock solution with water 1:10 and adding 0.15 N NH_4OH to a concentration of 5%.

The slides were stained for 8 min and rapidly dehydrated (30 sec/jar) in two changes of acetone and two changes of an acetone/xylol mixture (1:1). Finally, the slides were cleared in xylol.

For cytogenetic analysis metaphases of the marrow cells were scored for the presence of structural chromosome aberrations. Only cells with 35 or more chromosomes were scored. Metaphases with single or multiple aberrations were classified as abnormal. The types of visible aberrations scored were acentric fragments, dicentrics, ring and triradial translocations and abnormally long chromosomes.

RESULTS

The results are shown in Tables I-II and Fig. 6-8. Table I shows that only eight of 212 control metaphases examined had chromosome aberrations. The only types of aberrations that occurred in the controls were rings (8) and atypically long chromosomes (2). On the other hand, all three dosage groups showed aberrations in all categories of chromosome aberrations that were scored. 15.1% (0.15 mg/kg), 49.1% (0.4 mg/kg), and 73.6% (0.7 mg/kg) of the treated cells examined showed chromosomal damage. A total of 292 cells in the three dosage groups had the following types of abnormalities: 267 chromosome- and chromatid-type aberrations; 25 dicentrics; 105 rings; 11 triradial translocations and 27 abnormal long chromosomes. There was a marked difference in the percentage of abnormal cells between the four groups. As the dosage increased, the percentage of abnormal cells increased. However, the number of aberrations per cell did not vary much among the four groups.

Table II shows the range of the number of aberrations per cell. This was calculated at a student t confidence level of $p = 0.1$. A graphic representation of this is given in Fig. 6. It can be seen from this that the majority of the ranges of the four groups had a common value, and did not differ significantly.

Fig. 7 gives a pictorial aspect of mouse chromosomes in the bone marrow.

Fig. 8 is a graphic representation of the dose versus the percentage aberrant metaphases found for the different groups.

Table I

TEM-dose mg/kg	Np. of cells in metaphase examined	Cells with one or more chromosome aberration No.	%	Number of types of chromosome aberrations						
				Total number	Number/cell	Acentrics	Dicentrics	Rings	Triradials	Abnormal long
Control	212	8	3.8	10	1.25	0	0	8	0	2
0.15	212	32	15.1	42	1.31	20	3	13	1	5
0.4	212	104	49.1	153	1.47	101	9	29	5	9
0.7	212	156	73.6	232	1.49	146	13	55	5	13

Cytogenetic findings in mouse bone marrow after treatment with triethylenemelamine (TEM). Only metaphases with 35 or more chromosomes were scored. Mice were injected i.p. each day for 5 days with TEM. The controls did not receive TEM nor saline. Mice used for the different groups and control = B10.BR.

Table II

Dose TEM mg/kg	Range of values for no. of aberrations per cell[a]
Control	1.25 ± 0.48
0.15	1.31 ± 0.19
0.4	1.47 ± 0.12
0.7	1.49 ± 0.11

[a] p = 0.1

Upper and lower limits of values for the number of aberrations per cell. Three groups of mice (strain B10.BR) received differing dosages of triethylenemelamine (TEM) each day for 5 days. A control consisted of 10 untreated mice. Bone marrow from the femur was examined during metaphase arrest after colchicine was administered i.p. The total number of aberrations was divided by the number of abnormal cells for each group to arrive at the mean number. The upper and lower limits were determined by the standard error multiplied by the value for the group using the student t at a p value of 0.1.

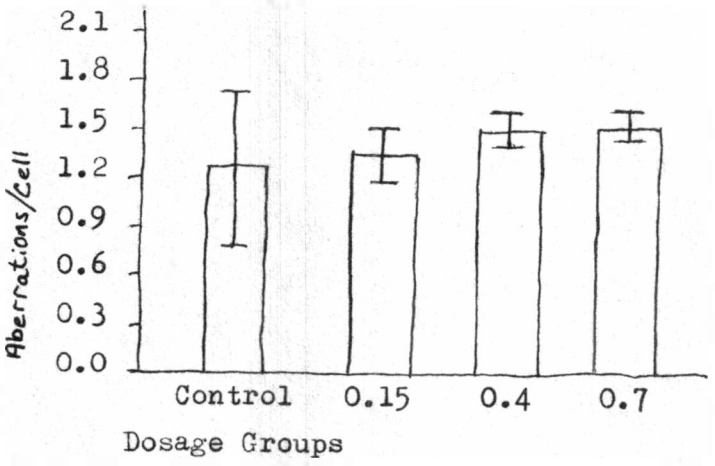

Figure 6. Graphic representation of the number of aberrations per cell. The thin "T" lines mark the upper and lower limits at a student t value of p = 0.1. The full bars represent the mean. This graph is based on the values shown in Table II.

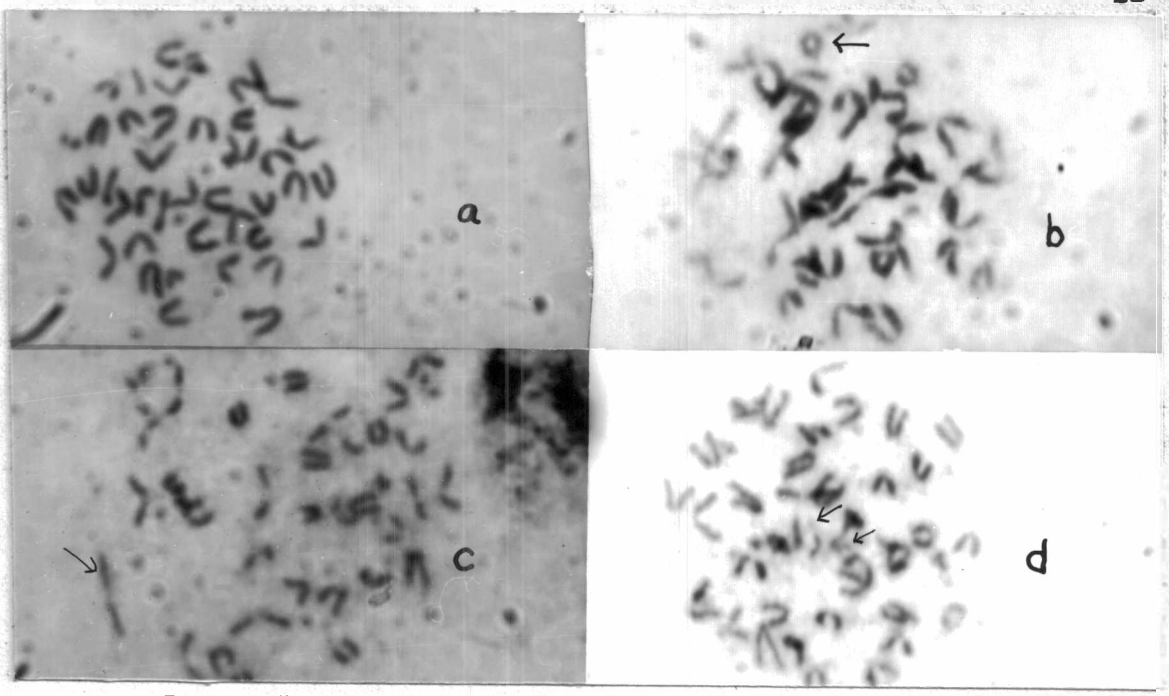

Figure 7. Photomicrographs showing cytogenetic studies on mouse bone marrow cell chromosomes after experimental animals were injected with triethylenemelamine (TEM). a, normal metaphase; b, rings; c, abnormal long chromosomes; d, acentric fragments.

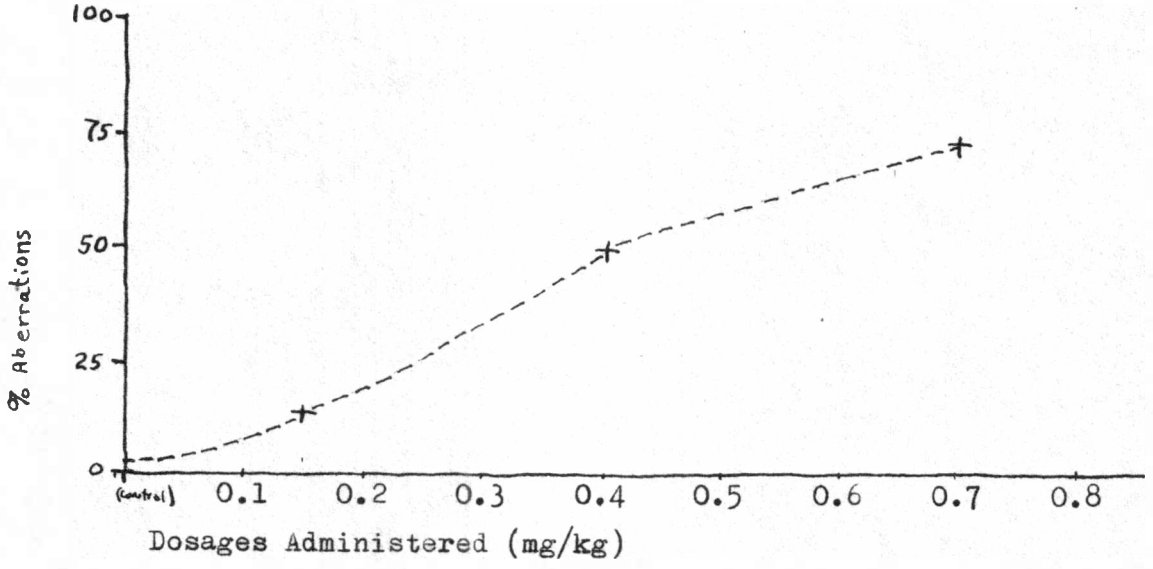

Figure 8. Dose-response curve based on percentage abnormal cells found during examination for visible chromosome aberrations after animals were treated with triethylenemelamine (TEM).

DISCUSSIONS AND CONCLUSIONS

From the results obtained three considerations must be made; 1) the change in the percentage of aberrant cells as the dose increases, 2) the change in the number of aberrations per cell as the dosage increases and 3) the change in the types of aberrations as the dosage increases.

The number of aberrant cells with increasing dosage is significant and near typical of other studies done with TEM. The work of others (Hitotsumachi, et al., 1977; Matter, et al., 1974; Matter, et al., 1975; Soares, et al., 1977; Weber, et al., 1975) deals with dominant-lethal, micronuclei, embryology and spermatogenesis studies. Although these differ from ours, there are general tendencies of the effects of TEM overall which allow some conclusions. Our results for dosages of 0.7 and 0.4 mg/kg (73.6% and 49.1% respectively) are in accordance with other studies done on TEM (Matter, et al., 1975). The near linearity of our results can be seen (Fig. 8), however the curve should be more of a shape described as a gradual upswinging. This is the general case with other experiments. This deviation could be due to the storage time of TEM (polymerization) and/or age and strain of the mice.

Table II and Fig. 6 show that the number of aberrations per cell is almost constant for all three dosage groups and control. This is not significant at a student t level of confidence $p = 0.1$ and would indicate that TEM does not act by attacking a single cell and its chromosomes, but rather by alkylating several different cells to the same degree of aberrancy.

Matter, et al., (1975) and our own studies show the major increase in structural aberrations was with acentric fragments. We also had a significant increase in the number of rings.

In summary, a dose-response relationship has been defined for cytogenetic studies involving the scoring of the number of aberrant cells, the number of aberrations per cell and the types of aberrations found in mouse bone marrow chromosomes after treatment with TEM. The importance of this dose-response relationship is that it permits the determination of a range of dosages from which a predictable responses can be deduced. This response to doses of TEM is important in determining the effective range so that it can be used in evaluation of similar pharmacologic or toxicologic substances and their activities.

Any contrast in our results from other TEM experiments may be due to variables of time storage of TEM, the batch of TEM, the age of our mice and/or the strain of mice used. Further experimentation in these areas (e.g., using mice of different ages to test for the effects of age on chromosome aberrations) would be warranted.

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