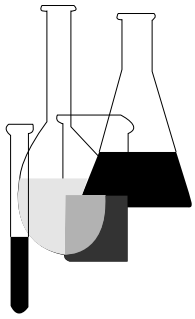
United States
Environmental Protection
Agency

Prevention, Pesticides
and Toxic Substances
(7101)

EPA 712–C–98–225
August 1998

Health Effects Test Guidelines OPPTS 870.5385 Mammalian Bone Marrow Chromosome Aberration Test



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S. Environmental Protection Agency under the Toxic Substances Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. 136, *et seq.*).

Final Guideline Release: This guideline is available from the U.S. Government Printing Office, Washington, DC 20402 on disks or paper copies: call (202) 512–0132. This guideline is also available electronically in PDF (portable document format) from EPA's World Wide Web site (http://www.epa.gov/epahome/research.htm) under the heading ''Researchers and Scientists/Test Methods and Guidelines/OPPTS Harmonized Test Guidelines.''

OPPTS 870.5385 Mammalian bone marrow chromosome aberration test.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*) and the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601).

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline are OPPT 40 CFR 798.5385 In vivo mammalian cytogenetics test: Bone marrow chromosomal analysis and OECD 475, Mammalian Bone Marrow Chromosome Aberration Test.

(b) **Purpose.** The mammalian *in vivo* chromosome aberration test is used for the detection of structural chromosome aberrations induced by test compounds in bone marrow cells of animals, usually rodents (see references in paragraphs (g)(1), (g)(2), (g)(3), and (g)(4) of this guideline). Structural chromosome aberrations may be of two types, chromosome or chromatid. An increase in polyploidy may indicate that a chemical has the potential to induce numerical aberrations. With the majority of chemical mutagens, induced aberrations are of the chromatid-type, but chromosome-type aberrations also occur. Chromosome mutations and related events are the cause of many human-genetic diseases and there is substantial evidence that chromosome mutations and related events causing alterations in oncogenes and tumour-suppressor genes are involved in cancer in humans and experimental systems.

(c) **Definitions.** The following definitions apply to the guideline:

Chromatid-type aberration is structural chromosome damage expressed as breakage of single chromatids or breakage and reunion between chromatids.

Chromosome-type aberration is structural chromosome damage expressed as breakage, or breakage and reunion, of both chromatids at an identical site.

Endoreduplication is a process in which after an S period of DNA replication, the nucleus does not go into mitosis but starts another S period. The result is chromosomes with 2,4,8,...chromatids.

Gap is an achromatic lesion smaller than the width of one chromatid, and with minimum misalignment of the chromatids.

Numerical aberration is a change in the number of chromosomes from the normal number characteristic of the animals utilized.

Polyploidy is a multiple of the haploid chromosome number (n) other than the diploid number (i.e., 3n, 4n, and so on).

Structural aberration is a change in chromosome structure detectable by microscopic examination of the metaphase stage of cell division, observed as deletions and fragments, intrachanges or interchanges.

(d) **Initial considerations.** (1) Rodents are routinely used in this test. Bone marrow is the target tissue in this test, since it is a highly vascularized tissue, and it contains a population of rapidly cycling cells that can be readily isolated and processed. Other species and target tissues are not the subject of this guideline.

(2) This chromosome aberration test is especially relevant to assessing mutagenic hazard in that it allows consideration of factors of *in vivo* metabolism, pharmacokinetics, and DNA-repair processes although these may vary among species and among tissues. An *in vivo* test is also useful for further investigation of a mutagenic effect detected by an *in vitro* test.

(3) If there is evidence that the test substance, or a reactive metabolite, will not reach the target tissue, it is not appropriate to use this test.

(e) **Test method**—(1) **Principle.** Animals are exposed to the test substance by an appropriate route of exposure and are sacrificed at appropriate times after treatment. Prior to sacrifice, animals are treated with a metaphase-arresting agent (e.g., colchicine or Colcemid®). Chromosome preparations are then made from the bone marrow cells and stained, and metaphase cells are analyzed for chromosome aberrations.

(2) **Description**—(i) **Preparations**—(A) **Selection of animal species.** Rats, mice, and Chinese hamsters are commonly used, although any appropriate mammalian species may be used. Commonly used laboratory strains of healthy young-adult animals should be employed. At the commencement of the study, the weight variation of animals should be minimal and not exceed ±20 percent of the mean weight of each sex.

(B) **Housing and feeding conditions.** The temperature in the experimental animal room should be 22 °C (±3 °C). Although the relative humidity should be at least 30 percent and preferably not exceed 70 percent other than during room cleaning, the aim should be 50–60 percent. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. The choice of diet may be influenced by the need to ensure a suitable admixture of a test substance when administered by this method. Animals may be housed individually, or be caged in small groups of the same sex.

(C) **Preparation of the animals.** Healthy, young-adult animals should be randomly assigned to the control and treatment groups. Cages should be arranged in such a way that possible effects due to cage placement

are minimized. The animals are identified uniquely. The animals are acclimated to the laboratory conditions for at least 5 days.

(D) **Preparation of doses.** Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted, if appropriate, prior to dosing of the animals. Liquid-test substances may be dosed directly or diluted prior to dosing. Fresh preparations of the test substance should be employed unless stability data demonstrate the acceptability of storage.

(ii) **Test conditions**—(A) **Solvent/vehicle.** The solvent/vehicle should not produce toxic effects at the dose levels used, and should not be suspected of chemical reaction with the test substance. If other than well-known solvents/vehicles are used, their inclusion should be supported with data indicating their compatibility. It is recommended that wherever possible, the use of an aqueous solvent/vehicle should be considered first.

(B) **Controls.** (*1*) Concurrent positive and negative (solvent/vehicle) controls should be included for each sex in each test. Except for treatment with the test substance, animals in the control groups should be handled in an identical manner to the animals in the treated groups.

(*2*) Positive controls should produce structural chromosome aberrations *in vivo* at exposure levels expected to give a detectable increase over background. Positive control doses should be chosen so that the effects are clear but do not immediately reveal the identity of the coded slides to the reader. It is acceptable that the positive control be administered by a route different from the test substance and sampled at only a single time. The use of chemical class related positive control chemicals may be considered, when available. Examples of positive control substances include:

Chemical	CAS number
Triethylenemelamine	[51–18–3]
Ethyl methanesulphonate	[62–50–0]
Ethyl nitrosourea	[759–73–9]
Mitomycin C	[50–07–7]
Cyclophosphamide (monohydrate)	[50–18–0] ([6055–19–2])

(*3*) Negative controls, treated with solvent or vehicle alone, and otherwise treated in the same way as the treatment groups, should be included for every sampling time, unless acceptable inter-animal variability and frequencies of cells with chromosome aberrations are available from historical control data. If single sampling is applied for negative controls, the most appropriate time is the first sampling time. In addition, untreated controls should also be used unless there are historical or published control data demonstrating that no deleterious or mutagenic effects are induced by the chosen solvent/vehicle.

(3) **Procedure**—(i) **Number and sex of animals.** Each treated and control group should include at least five analyzable animals per sex. If at the time of the study there are data available from studies in the same species and using the same route of exposure that demonstrate that there are no substantial differences in toxicity between sexes, then testing in a single sex will be sufficient. Where human exposure to chemicals may be sex specific, as for example with some pharmaceutical agents, the test should be performed with animals of the appropriate sex.

(ii) **Treatment schedule.** (A) Test substances are preferably administered as a single treatment. Test substances may also be administered as a split dose, i.e. two treatments on the same day separated by no more than a few hours, to facilitate administering a large volume of material. Other dose regimens should be scientifically justified.

(B) Samples should be taken at two separate times following treatment on one day. For rodents, the first sampling interval is 1.5 normal cell-cycle length (the latter being normally 12–18 hours) following treatment. Since the time required for uptake and metabolism of the test substance as well as its effect on cell-cycle kinetics can affect the optimum time for chromosome aberration detection, a later sample collection 24 hours after the first sample time is recommended. If dose regimens of more than 1 day are used, one sampling time at 1.5 normal cell-cycle lengths after the final treatment should be used.

(C) Prior to sacrifice, animals should be injected intraperitoneally with an appropriate dose of a metaphase arresting agent (e.g., Colcemid® or colchicine). Animals are sampled at an appropriate interval thereafter. For mice this interval is approximately 3–5 hours; for Chinese hamsters this interval is approximately 4–5 hours. Cells should be harvested from the bone marrow and analysed from chromosome aberrations.

(iii) **Dose levels.** If a range finding study is performed because there are no suitable data available, it should be performed in the same laboratory, using the same species, strain, sex, and treatment regimen to be used in the main study (see reference in paragraph (g)(5) of this guideline). If there is toxicity, three-dose levels should be used for the first sampling time. These dose levels should cover a range from the maximum to little or no toxicity. At the later sampling time only the highest dose needs to be used. The highest dose is defined as the dose producing signs of toxicity such that higher dose levels, based on the same dosing regimen, would be expected to produce lethality. Substances with specific biological activities at low non-toxic doses (such as hormones and mitogens) may be exceptions to the dose-setting criteria and should be evaluated on a case-by-case basis. The highest dose may also be defined as a dose that produces some indication of toxicity in the bone marrow (e.g., greater than 50 percent reduction in mitotic index).

(iv) **Limit test.** If a test at one dose level of at least 2,000 mg/kg body weight using a single treatment, or as two treatments on the same day, produces no observable toxic effects, and if genotoxicity would not be expected based on data from structurally related compounds, then a full study using three-dose levels may not be considered necessary. For studies of a longer duration, the limit dose is 2,000 mg/kg/body weight/ day for treatment up to 14 days, and 1,000 mg/kg/body weight/day for treatment longer than 14 days. Expected human exposure may indicate the need for a higher-dose level to be used in the limit test.

(v) **Administration of doses.** The test substance is usually administered by gavage using a stomach tube or a suitable intubation cannula, or by intraperitoneal injection. Other routes of exposure may be acceptable where they can be justified. The maximum volume of liquid that can be administered by gavage or injection at one time depends on the size of the test animal. The volume should not exceed 2 ml/100g body weight. The use of volumes higher than these must be justified. Except for irritating or corrosive substances which will normally reveal exacerbated effects with higher concentrations, variability in test volume should be minimised by adjusting the concentration to ensure a constant volume at all dose levels.

(vi) **Chromosome preparation.** Immediately after sacrifice, bone marrow should be obtained, exposed to hypotonic solution and fixed. The cells should be then spread on slides and stained.

(vii) **Analysis.** (A) The mitotic index should be determined as a measure of cytotoxicity in at least 1,000 cells per animal for all treated animals (including positive controls) and untreated negative control animals.

(B) At least 100 cells should be analyzed for each animal. This number could be reduced when high numbers of aberrations are observed. All slides, including those of positive and negative controls, should be independently coded before microscopic analysis. Since slide preparation procedures often result in the breakage of a proportion of metaphases with loss of chromosomes, the cells scored should therefore contain a number of centromeres equal to the number 2n ±2.

(f) **Data and reporting**—(1) **Treatment of results.** Individual animal data should be presented in tabular form. The experimental unit is the animal. For each animal the number of cells scored, the number of aberrations per cell and the percentage of cells with structural chromosome aberration(s) should be evaluated. Different types of structural chromosome aberrations should be listed with their numbers and frequencies for treated and control groups. Gaps should be recorded separately and reported but generally not included in the total aberration frequency. If there is no evidence for a difference in response between the sexes, the data may be combined for statistical analysis.

(2) **Evaluation and interpretation of results.** (i) There are several criteria for determining a positive result, such as a dose-related increase in the relative number of cells with chromosome aberrations or a clear increase in the number of cells with aberrations in a single-dose group at a single-sampling time. Biological relevance of the results should be considered first. Statistical methods may be used as an aid in evaluating the test results (see reference in paragraph (g)(6) of this guideline). Statistical significance should not be the only determining factor for a positive response. Equivocal results should be clarified by further testing preferably using a modification of experimental conditions.

(ii) An increase in polyploidy may indicate that the test substance has the potential to induce numerical chromosome aberrations. An increase in endoreduplication may indicate that the test substance has the potential to inhibit cell-cycle progression (see references in paragraphs (g)(7) and (g)(8) of this guideline).

(iii) A test substance for which the results do not meet the criteria in paragraphs (f)(2)(i) and (f)(2)(ii) of this guideline is considered non-mutagenic in this test.

(iv) Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance. Results may remain equivocal or questionable regardless of the number of experiments performed.

(v) Positive results from the *in vivo* chromosome aberration test indicate that a substance induces chromosome aberrations in the bone marrow of the species tested. Negative results indicate that, under the test conditions, the test substance does not induce chromosome aberrations in the bone marrow of the species tested.

(vi) The likelihood that the test substance or its metabolites reach the general circulation or specifically the target tissue (e.g., systemic toxicity) should be discussed.

(3) **Test report.** The test report should include the following information:

(i) Test substance:

(A) Identification data and CAS No., if known.

(B) Physical nature and purity.

(C) Physicochemical properties relevant to the conduct of the study.

(D) Stability of the test substance, if known.

(ii) Solvent/vehicle:

(A) Justification for choice of vehicle.

(B) Solubility and stability of the test substance in solvent/vehicle, if known.

(iii) Test animals:

(A) Species/strain used.

(B) Number, age, and sex of animals.

(C) Source, housing conditions, diet, etc.

(D) Individual weight of the animals at the start of the test, including body weight range, mean, and standard deviation for each group.

(iv) Test conditions:

(A) Positive and negative (vehicle/solvent) controls.

(B) Data from range-finding study, if conducted.

(C) Rationale for dose level selection.

(D) Details of test substance preparation.

(E) Details of the administration of the test substance.

(F) Rationale for route of administration.

(G) Methods for verifying that the test substance reached the general circulation or target tissue, if applicable.

(H) Conversion from diet/drinking water test substance concentration parts per million (ppm) to the actual dose (mg/kg body weight/day), if applicable.

(I) Details of food and water quality.

(J) Detailed description of treatment and sampling schedules.

(K) Methods for measurement of toxicity.

(L) Identity of metaphase arresting substance, its concentration and duration of treatment.

(M) Methods of slide preparation.

(N) Criteria for scoring aberrations.

(O) Number of cells analysed per animal.

(P) Criteria for considering studies as positive, negative or equivocal.

(v) Results:

(A) Signs of toxicity.

(B) Mitotic index.

(C) Type and number of aberrations, given separately for each animal.

(D) Total number of aberrations per group with means and standard deviations.

(E) Number of cells with aberrations per group with means and standard deviations.

(F) Changes in ploidy, if seen.

(G) Dose-response relationship, where possible.

(H) Statistical analyses, if any.

(I) Concurrent negative control data.

(J) Historical negative control data with ranges, means and standard deviations.

(K) Concurrent positive control data.

(vi) Discussion of the results.

(vii) Conclusion.

(g) **References.** The following references should be consulted for additional background information on this test guideline.

(1) Adler, I.D. Cytogenetic Tests in Mammals. Mutagenicity Testing: A Practical Approach. (Eds.) S. Venitt and J.M. Parry (IRL Press, Oxford, Washington DC, pp. 275–306 (1984).

(2) Preston, R.J. et al. Mammalian *In Vivo* Cytogenetic Assays: Analysis of Chromosome Aberrations in Bone Marrow Cells. *Mutation Research* 189, 157–165 (1987).

(3) Richold, M. et al. *In Vivo* Cytogenetic Assays. (Ed.) D.J. Kirkland. Basic Mutagenicity Tests, UKEMS Recommended Procedures. UKEMS Subcommittee on Guidelines for Mutagenicity Testing. Report. Part I revised. (Cambridge University Press, Cambridge, New York, Port Chester, Melbourne, Sydney, pp. 115–141 (1990).

(4) Tice, R.R. et al. Report from the Working Group on the *In Vivo* Mammalian Bone Marrow Chromosomal Aberration Test. *Mutation Research* 312, 305–312 (1994).

(5) Fielder, R.J. et al. Report of British Toxicology Society/UK Environmental Mutagen Society Working Group: Dose Setting in *In Vivo* Mutagenicity Assays. *Mutagenesis* 7, 313–319 (1992).

(6) Lovell, D.P. et al. Statistical Analysis of *In Vivo* Cytogenetic Assays. UKEMS Sub-Committee on Guidelines for Mutagenicity Testing. Report Part III. Statistical Evaluation of Mutagenicity Test Data. (Ed.) D. J. Kirkland (Cambridge University Press, Cambridge, pp. 184–232 (1989).

(7) Locke-Huhle, C. Endoreduplication in Chinese Hamster Cells During Alpha-Radiation Induced G2 Arrest. *Mutation Research* 119, 403–413 (1983).

(8) Huang, Y. et al. Aphidicolin-Induced Endoreduplication in Chinese Hamster Cells. *Cancer Research* 43, 1362–1364 (1983).