

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of structural chromosomal aberrations or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) *Test evaluation.* (i) Positive results in the *in vitro* cytogenetics assay indicate that under the test conditions the test substance induces chromosomal aberrations in cultured mammalian somatic cells.

(ii) Negative results indicate that under the test conditions the test substance does not induce chromosomal aberrations in cultured mammalian somatic cells.

(5) *Test report.* In addition to the reporting recommendations as specified under 40 CFR part 792, subpart J the following specific information shall be reported:

(i) Cells used, density and passage number at time of treatment, number of cell cultures.

(ii) Methods used for maintenance of cell cultures including medium, temperature and CO₂ concentration.

(iii) Test chemical vehicle, concentration and rationale for the selection of the concentrations used in the assay, duration of treatment.

(iv) Details of both the protocol used to prepare the metabolic activation system and of its use in the assay.

(v) Identity of spindle inhibitor, its concentration and duration of treatment.

(vi) Date of cell harvest.

(vii) Positive and negative controls.

(viii) Methods used for preparation of slides for microscopic examination.

(ix) Number of metaphases analysed.

(x) Mitotic index where applicable.

(xi) Criteria for scoring aberrations.

(xii) Type and number of aberrations, given separately for each treated and control culture, total number of aberrations per group; frequency distribution of number of chromosomes in established cell lines and strains.

(xiii) Dose-response relationship, if applicable.

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

(1) Ames, B.N., McCann, J., Yamasaki, E. "Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test," *Mutation Research*, 31:347-364 (1975).

(2) Evans, H.J. "Cytological methods for detecting chemical mutagens," *Chemical mutagens, principles and methods for their detection*, Vol. 4, Ed. A. Hollaender (New York, London: Plenum Press, 1976) pp. 1-29.

(3) Howard, P.N., Bloom, A.D., Krooth, R.S. "Chromosomal aberrations induced by N-methyl-N'-nitro-N-nitrosoguanidine in mammalian cells," *In Vitro* 7:359-365 (1972).

(4) Ishidate, M. Jr., Odashima, S. "Chromosome tests with 134 compounds on Chinese hamster cells *in vitro*: A screening for chemical carcinogens," *Mutation Research*, 48:337-354 (1975).

(5) Preston, R.J., Au, W., Bender, M.A., Brewen, J.G., Carrano, A.V., Heddle, J.A., McFee, A.F., Wolff, S., Wassom, J.S., "Mammalian *in vivo* and *in vitro* cytogenetic assays: A report of the Gene-tox Program," *Mutation Research*, 87:143-188 (1981).

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19079, May 20, 1987]

§ 798.5385 *In vivo* mammalian bone marrow cytogenetics tests: Chromosomal analysis.

(a) *Purpose.* The *in vivo* bone marrow cytogenetic test is a mutagenicity test for the detection of structural chromosomal aberrations. Chromosomal aberrations are generally evaluated in first post-treatment mitoses. With the majority of chemical mutagens, induced aberrations are of the chromatid type but chromosome type aberrations also occur.

(b) *Definitions.* (1) Chromosome-type aberrations are changes which result from damage expressed in both sister chromatids at the same time.

(2) Chromatid-type aberrations are damage expressed as breakage of single chromatids or breakage and/or reunion between chromatids.

(c) *Reference substances.* Not applicable.

(d) *Test method—(1) Principle.* Animals are exposed to test chemicals by appropriate routes and are sacrificed at sequential intervals. Chromosome preparations are made from bone marrow cells. The stained preparations are examined and metaphase cells are scored for chromosomal aberrations.

(2) *Description.* The method employs bone marrow of laboratory rodents which have been exposed to test chemicals. Prior to sacrifice, animals are further treated with a spindle inhibitor, (e.g., colchicine or Colcemid®) to arrest the cells in c-metaphase. Chromosome preparations from the cells are stained and scored for chromosomal aberrations.

(3) *Animal selection—(i) Species and strain.* Any appropriate mammalian species may be used. Examples of commonly used rodent species are rats, mice, and hamsters.

(ii) *Age.* Healthy young adult animals shall be used.

(iii) *Number and sex.* At least five female and five male animals per experimental and control group shall be used. Thus, 10 animals would be sacrificed per time per group treated with the test compound if several test times after treatment are included in the experimental schedule. The use of a single sex or smaller number of animals should be justified.

(iv) *Assignment to groups.* Animals shall be randomized and assigned to treatment and control groups.

(4) *Control groups—(1) Concurrent controls.* (i) Concurrent positive and negative (vehicle) controls shall be included in the assay.

(ii) *Positive controls.* A single dose positive control showing a significant response at any one time point is adequate. A compound known to produce chromosomal aberrations in vivo shall be employed as the positive control.

(5) *Test chemicals—(i) Vehicle.* When possible, test chemicals shall be dissolved in isotonic saline or distilled water. Water insoluble chemicals may be dissolved or suspended in appropriate vehicles. The vehicles used shall neither interfere with the test chemical nor produce toxic effects. Fresh

preparations of the test compound should be employed.

(ii) *Dose levels.* For an initial assessment, one dose of the test substance may be used, the dose being the maximum tolerated dose (to a maximum of 5,000 mg/kg) or that producing some indication of cytotoxicity (e.g., partial inhibition of mitosis) or shall be the highest dose attainable (to a maximum of 5,000 mg/kg). Additional dose levels may be used. For determination of dose-response, at least three dose levels should be used.

(iii) *Route of administration.* The usual routes are oral or by intraperitoneal injection. Other routes may be appropriate.

(iv) *Treatment schedule.* In general, test substances should be administered once only. However, based on toxicological information a repeated treatment schedule may be employed.

(e) *Test performance—(1) Generally the test may be performed in two assays.* (i) Animals should be treated with the test substance once at the selected dose(s). Samples should be taken at three times after treatment. For rodents, the central sampling interval is 24 hours. Since cell cycle kinetics can be influenced by the test substance, one earlier and one later sampling interval adequately spaced within the range of 6 to 48 hours shall be applied. Where the additional dose levels are tested in a subsequent experiment, samples shall be taken at the predetermined most sensitive interval or, if this is not established, at the central sampling time. If the most sensitive interval is known and documented with data, only this one time point shall be sampled.

(ii) If a repeated treatment schedule is used at the selected dose(s), samples shall be taken 6 and 24 hours after the last treatment; other sampling times may be used if justified. Where the additional dose levels are tested in a subsequent experiment, samples shall be taken at the predetermined most sensitive interval or, if this is not established, at 6 hours after the last treatment.

(2) *Administration of spindle inhibitor.* Prior to sacrifice, animals shall be injected IP with an appropriate dose of a spindle inhibitor (e.g., colchicine or

Colcemid®) to arrest cells in c-metaphase.

(3) *Preparation of slides.* Immediately after sacrifice, the bone marrow shall be obtained, exposed to hypotonic solution, and fixed. The cells shall then be spread on slides and stained. Chromosome preparations shall be made following standard procedures.

(4) *Analysis.* The number of cells to be analyzed per animal should be based upon the number of animals used, the negative control frequency, the predetermined sensitivity, and the power chosen for the test. Slides shall be coded before microscopic analysis.

(f) *Data and report*—(1) *Treatment of results.* Data should be presented in tabular form for both cells and animals. Different types of structural chromosomal aberrations should be listed with their numbers and a mean frequency per cell for each animal in all treated and control groups. Gaps (achromatic lesions) should be recorded separately and not included in the total aberration frequency. Differences among animals within each group should be considered before making comparisons between treated and control groups.

(2) *Statistical evaluation.* Data should be evaluated by appropriate statistical methods.

(3) *Interpretation of results.* (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of structural chromosomal aberrations or abnormal metaphase figures. Another criterion may be based upon detection of a reproducible and statistically significant positive response for a least one of the test points.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of chromosomal aberrations or abnormal metaphase figures or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) *Test evaluation.* (i) Positive results in the *in vivo* bone marrow cyto-

genetics assay indicate that under the test conditions the test substance induces chromosomal aberrations in the bone marrow of the test species.

(ii) Negative results indicate that under the test conditions, the test substance does not induce chromosomal aberrations in the bone marrow of the test species.

(5) *Test report.* In addition to the reporting recommendations as specified under 40 CFR part 792, subpart J the following specific information shall be reported:

(i) Species, strain, age, weight, number and sex of animals in each treatment and control group.

(ii) Test chemical vehicle, dose levels used, rationale for dose selection.

(iii) Route of administration, treatment and sampling schedules, toxicity data, negative and positive controls.

(iv) Identity of spindle-inhibitor, its concentration and duration of treatment.

(v) Details of the protocol used for chromosome preparation, number of cells scored per animal, type and number of aberrations given separately for each treated and control animal.

(vi) Mitotic index, where applicable.

(vii) Criteria for scoring aberrations.

(viii) Number and frequency of aberrant cells per animal in each treatment and control groups.

(ix) Total number of aberrations per group.

(x) Number of cells with aberrations per group.

(xi) Dose-response relationship, if applicable.

(g) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Adler, I.D., Ramarao, G., Epstein, S.S. "In vivo cytogenetic effects of trimethyl-phosphate and of TEPA on bone marrow cells of male rats," *Mutation Research*, 13:263-273 (1971).

(2) Evans, H.J. "Cytological methods for detecting chemical mutagens," *Chemical Mutagens: Principles and Methods for Their Detection*, Vol. 4. Ed. A. Hollaender (New York and London: Plenum Press, 1976) pp. 1-29.

(3) Kilian, J.D., Moreland, F.E. Bengel, M.C., Legator, M.S., Whorton, E.B. Jr. "A collaborative study to

measure intralaboratory variation with the *in vivo* bone marrow metaphase procedure," *Handbook of mutagenicity test procedures*. Eds. Kilby, B.J., Legator, M. Nichols, C., Ramel, D., (Amsterdam: Elsevier/North Holland Biomedical Press, 1977) 243-260.

(4) Preston, J.R., Au, W., Bender, M.A., Brewen, J.G., Carrano, A.V., Heddle, J.A., McFee, A.F., Wolff, S., Wassom, J. "Mammalian *in vivo* and *in vitro* cytogenetics assays: Report of the Gene-Tox Program," *Mutation Research*, 87:143-188 (1981).

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19080, May 20, 1987]

§ 798.5395 *In vivo* mammalian bone marrow cytogenetics tests: Micronucleus assay.

(a) *Purpose*. The micronucleus test is a mammalian *in vivo* test which detects damage of the chromosomes or mitotic apparatus by chemicals. Polychromatic erythrocytes in the bone marrow of rodents are used in this assay. When the erythroblast develops into an erythrocyte the main nucleus is extruded and may leave a micronucleus in the cytoplasm. The visualization of micronuclei is facilitated in these cells because they lack a nucleus. Micronuclei form under normal conditions. The assay is based on an increase in the frequency of micronucleated polychromatic erythrocytes in bone marrow of treated animals.

(b) *Definition*. Micronuclei are small particles consisting of acentric fragments of chromosomes or entire chromosomes, which lag behind at anaphase of cell division. After telophase, these fragments may not be included in the nuclei of daughter cells and form single or multiple micronuclei in the cytoplasm.

(c) *Reference substances*. Not applicable.

(d) *Test method*—(1) *Principle*. (i) Animals are exposed to test substance by an appropriate route. They are sacrificed, the bone marrow extracted and smear preparations made and stained. Polychromatic erythrocytes are scored for micronuclei under the microscope.

(ii) Micronuclei may also be detected in other test systems:

- (A) Tissue culture.
- (B) Plants.

(C) Blood smears.

(D) Fetal tissues.

(E) Meiotic cells.

(F) Hepatic cells.

(iii) The present guideline is based on the mammalian bone marrow assay.

(2) *Description*. The method employs bone marrow of laboratory mammals which are exposed to test substances.

(3) *Animal selection*—(i) *Species and strain*. Mice are recommended. However, any appropriate mammalian species may be used.

(ii) *Age*. Young adult animals shall be used.

(iii) *Number and sex*. At least five female and five male animals per experimental and control group shall be used. Thus, 10 animals would be sacrificed per time per group if several test times after treatment were included in the experimental schedule. The use of a single sex or a smaller number of animals should be justified.

(iv) *Assignment to groups*. Animals shall be randomized and assigned to treatment and control groups.

(4) *Control groups*—(i) *Concurrent controls*. Concurrent positive and negative (vehicle) controls shall be included in each assay.

(ii) *Positive controls*. A compound known to produce micronuclei *in vivo* shall be employed as the positive control.

(5) *Test chemicals*—(i) *Vehicle*. When appropriate for the route of administration, solid and liquid test substances should be dissolved or suspended in distilled water or isotonic saline. Water insoluble chemicals may be dissolved or suspended in appropriate vehicles. The vehicle used shall neither interfere with the test compound nor produce toxic effects. Fresh preparations of the test compound should be employed.

(ii) *Dose levels*. For an initial assessment, one dose of the test substance may be used, the dose being the maximum tolerated dose (to a maximum of 5,000 mg/kg) or that producing some indication of cytotoxicity, e.g., a change in the ratio of polychromatic to normochromatic erythrocytes. Additional dose levels may be used. For determination of dose response, at least three dose levels shall be used.