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[59 FR 33093, June 27, 1994, as amended at 61 FR 36513, July 11, 1996]

§ 79.64 *In vivo* micronucleus assay.

(a) *Purpose.* The micronucleus assay is an *in vivo* cytogenetic test which uses erythrocytes in the bone marrow of rodents to detect chemical damage to the chromosomes or mitotic apparatus of mammalian cells. As the erythroblast develops into an erythrocyte (red blood cell), its main nucleus is extruded and may leave a micronucleus in the cell body; a few micronuclei form under normal conditions in blood elements. This assay is based on an increase in the frequency of micronucleated erythrocytes found in bone marrow from treated animals compared to that of control animals. The visualization of micronuclei is facilitated in these cells because they lack a main nucleus.

(b) *Definitions.* For the purposes of this section the following definitions apply:

Micronuclei mean 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.

Polychromatic erythrocyte (PCE) means an immature red blood cell that, because it contains RNA, can be differentiated by appropriate staining techniques from a normochromatic erythrocyte (NCE), which lacks RNA. In one to two days, a PCE matures into a NCE.

(c) *Test method*—(1) *Principle of the test method.* (i) Groups of rodents are exposed by the inhalation route for a minimum of 6 hours/day over a period of not less than 28 days to three or more concentrations of a test substance in air. Groups of animals are sacrificed at the end of the exposure

period and femoral bone marrow is extracted. The bone marrow is then smeared onto glass slides, stained, and PCEs are scored for micronuclei. Researchers may need to run a trial at the highest tolerated concentration of the test atmosphere to optimize the sample collection time for micronucleated cells.

(ii) This assay may be done separately or in combination with the subchronic toxicity study, pursuant to the provisions in § 79.62.

(2) *Species and strain.* (i) The rat is the recommended test animal. Other rodent species may be used in this assay, but use of that species will be justified by the tester.

(ii) If a strain of mouse is used in this assay, the tester shall sample peripheral blood from an appropriate site on the test animal, e.g., the tail vein, as a source of normochromatic erythrocytes. Results shall be reported as outlined later in this guideline with "normochromatic" interchanged for "polychromatic", where specified.

(3) *Animal number and sex.* At least five female and five male animals per experimental/sample and control group shall be used. The use of a single sex or a smaller number of animals shall be justified.

(4) *Positive control group.* A single concentration of a compound known to produce micronuclei *in vivo* is adequate as a positive control if it shows a significant response at any one time point; additional concentration levels may be used. To select an appropriate concentration level, a pilot or trial study may be advisable. Initially, one concentration of the test substance may be used, the maximum tolerated dose or that producing some indication of toxicity, e.g., a drop in the ratio of polychromatic to normochromatic erythrocytes. Intraperitoneal injection of 1,2-dimethyl-benz-anthracene or benzene are examples of positive control exposures. A concentration of 50-80 percent of an LD50 may be a suitable guide.

(d) *Test performance*—(1) *Inhalation exposure.* (i) All data developed within this study shall be in accordance with good laboratory practice provisions under § 79.60.

(ii) The general conduct of this study shall be in accordance with the vehicle emissions inhalation exposure guideline in § 79.61.

(2) *Preparation of slides and sampling times.* Within twenty-four hours of the last exposure, test animals will be sacrificed. One femur from each test animal will be removed and placed in fetal bovine serum. The bone marrow is removed, cells processed, and two bone marrow smears are made for each animal on glass microscope slides. The slides are stained with acridine-orange (AO) or another appropriate stain (Giemsa + Wright's, etc.) and examined under a microscope.

(3) *Analysis.* Slides shall be coded for study before microscopic analysis. At least 1,000 first-division erythrocytes per animal shall be scored for the incidence of micronuclei. Sexes will be analyzed separately.

(e) *Data and report*—(1) *Treatment of results.* In addition to the reporting requirements specified under §§ 79.60 and 79.61, the final test report must include the criteria for scoring micronuclei. Individual data shall be presented in a tabular form including both positive and negative controls and experimental groups. The number of polychromatic erythrocytes scored, the number of micronucleated erythrocytes, the percentage of micronucleated cells, and, where applicable, the percentage of micronucleated erythrocytes shall be listed separately for each experimental and control animal. Absolute numbers shall be included if percentages are reported.

(2) *Interpretation of data.* (i) There are several criteria for determining a positive response, one of which is a statistically significant dose-related increase in the number of micronucleated polychromatic erythrocytes. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test substance concentrations.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of micronucleated polychromatic erythrocytes or a statistically significant and reproducible positive response

at any one of the test points is considered nonmutagenic in this system.

(3) *Test evaluation.* (i) Positive results in the micronucleus test provide information on the ability of a chemical to induce micronuclei in erythrocytes of the test species under the conditions of the test. This damage may have been the result of chromosomal damage or damage to the mitotic apparatus.

(ii) Negative results indicate that under the test conditions the test substance does not produce micronuclei in the bone marrow of the test species.

(f) *Test report.* In addition to the reporting recommendations as specified under § 79.60, the following specific information shall be reported:

(1) Test atmosphere concentration(s) used and rationale for concentration selection.

(2) Rationale for and description of treatment and sampling schedules, toxicity data, negative and positive controls.

(3) Historical control data (negative and positive), if available.

(4) Details of the protocol used for slide preparation.

(5) Criteria for identifying micronucleated erythrocytes.

(6) Micronucleus analysis by animal and by group for each concentration (sexes analyzed separately).

(i) Ratio of polychromatic to normochromatic erythrocytes.

(ii) Number of polychromatic erythrocytes with micronuclei.

(iii) Number of polychromatic erythrocytes scored.

(7) Statistical methodology chosen for test analysis.

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

(1) 40 CFR 798.5395, *In Vivo*, Mammalian Bone Marrow Cytogenetics Tests: Micronucleus Assay.

(2) Cihak, R. "Evaluation of Benzidine by the Micronucleus Test." *Mutation Research*, 67: 383-384 (1979).

(3) 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.

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(4) Heddle, J.A., *et al.* "The Induction of Micronuclei as a Measure of Genotoxicity. A Report of the U.S. Environmental Protection Agency Gene-Tox Program." *Mutation Research*, 123:61-118 (1983).

(5) Preston, J.R. *et al.* "Mammalian *In Vivo* and *In Vitro* Cytogenetics Assays: Report of the Gene-Tox Program." *Mutation Research*, 87:143-188 (1981).

(6) Schmid, W. "The micronucleus test for cytogenetic analysis", *Chemical Mutagens, Principles and Methods for their Detection*. Vol. 4 Hollaender A, (Ed. A ed. (New York and London: Plenum Press, (1976) pp. 31-53.

(7) Tice, R.E., and Al Pellom "User's guide: Micronucleus assay data management and analysis system", NTIS Order no. PB-90-212-598AS.

§ 79.65 *In vivo* sister chromatid exchange assay.

(a) *Purpose.* The *in vivo* sister chromatid exchange (SCE) assay detects the ability of a chemical to enhance the exchange of DNA between two sister chromatids of a duplicating chromosome. The most commonly used assays employ mammalian bone marrow cells or peripheral blood lymphocytes, often from rodent species.

(b) *Definitions.* For the purposes of this section, the following definitions apply:

C-metaphase means a state of arrested cell growth typically seen after treatment with a spindle inhibitor, *i.e.*, colchicine.

Sister chromatid exchange means a reciprocal interchange of the two chromatid arms within a single chromosome. This exchange is visualized during the metaphase portion of the cell cycle and presumably requires the enzymatic incision, translocation and ligation of at least two DNA helices.

(c) *Test method*—(1) *Principle of the test method.* (i) Groups of rodents are exposed by the inhalation route for a minimum of 6 hours/day over a period of not less than 28 days to three or more concentrations of a test substance in air. Groups of animals are sacrificed at the end of the exposure period and blood lymphocyte cell cultures are prepared from study animals. Cell growth is suspended after a time

and cells are harvested, fixed and stained before scoring for SCEs. Researchers may need to run a trial at the highest tolerated concentration of the test atmosphere to optimize the sample collection time for second division metaphase cells.

(ii) This assay may be done separately or in combination with the subchronic toxicity study, pursuant to the provisions in § 79.62.

(2) *Description.* (i) The method described here employs peripheral blood lymphocytes (PBL) of laboratory rodents exposed to the test atmosphere.

(ii) Within twenty-four hours of the last exposure, test animal lymphocytes are obtained by heart puncture and duplicate cell cultures are started for each animal. Cultures are grown in bromo-deoxyuridine (BrdU), and then a spindle inhibitor (e.g., colchicine) is added to arrest cell growth. Cells are harvested, fixed, and stained and their chromosomes are scored for SCEs.

(3) *Species and strain.* The rat is the recommended test animal. Other rodent species may be used in this assay, but use of that species will be justified by the tester.

(4) *Animal number and sex.* At least five female and five male animals per experimental and control group shall be used. The use of a single sex or different number of animals shall be justified.

(5) *Positive control group.* A single concentration of a compound known to produce SCEs *in vivo* is adequate as a positive control if it shows a significant response at any one time point; additional concentration levels may be used. To select an appropriate concentration level, a pilot or trial study may be advisable. Initially, one concentration of the test substance may be used, the maximum tolerated dose or that producing some indication of toxicity as evidenced by animal morbidity (including death) or target cell toxicity. Intraperitoneal injection of 1,2-dimethyl-benz-anthracene or benzene are examples of positive control exposures. A concentration of 50-80 percent of an LD50 would also be a suitable guide.

(6) *Inhalation exposure.* (i) All data developed within this study shall be in