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[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.

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

(iv) *Treatment schedule.* Test substances should generally be administered only once. However, based upon toxicological information a repeated treatment schedule may be employed.

(e) *Test performance*—(1) *Treatment and sampling times.* (i) Animals shall be treated with the test substance once at the highest tolerated dose. Sampling times should coincide with the maximum responses of the assay which varies with the test substance. Therefore, using the highest dose, bone marrow samples should be taken at least three times, starting not earlier than 12 hours after treatment, with appropriate intervals following the first sample but not extending beyond 72 hours. When other doses are used sampling shall be at the maximum sensitive period, or, if that is not known, approximately 24 hours after treatment. Other appropriate sampling times may be used in addition. If the most sensitive interval is known and documented with data, only this one time point need be sampled.

(ii) If a repeated treatment schedule is used, samples shall be taken at least three times, starting not earlier than 12 hours after the last treatment and at appropriate intervals following the first sample, but not extending beyond 72 hours.

(iii) Bone marrow shall be obtained immediately after sacrifice. Cells shall be prepared, put on slides, spread as a smear and stained.

(2) *Analysis.* Slides shall be coded before microscopic analysis. At least 1,000 polychromatic erythrocytes per animal shall be scored for the incidence of micronuclei. The ratio of polychromatic to normochromatic erythrocytes should be determined for each animal by counting a total of 200 erythrocytes. To ensure consistency with OECD and other guidelines, 1,000 polychromatic erythrocytes are recommended. Additional information may be obtained by scoring normochromatic erythrocytes for micronuclei.

(f) *Data and report*—(1) *Treatment of results.* Criteria for scoring micronuclei shall be given. Individual data shall be presented in a tabular form including positive and negative (vehicle) controls and experimental groups. The number of polychromatic erythrocytes scored, the number of micronucleated polychromatic erythrocytes, the percentage of micronucleated cells, the number of micronucleated normochromatic erythrocytes, and, if applicable, the percentage of micronucleated erythrocytes and the ratio of normochromatic to polychromatic erythrocytes shall be listed separately for each experimental and control animal. Absolute numbers shall be included if percentages are reported.

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

(3) *Interpretation of results.* (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.

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

(4) *Test evaluation.* (i) The results of the micronucleus test provide information on the ability of a chemical to induce micronuclei in polychromatic 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.

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

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

(v) Criteria for identifying micronucleated erythrocytes.

(vi) Dose-response relationship, if applicable.

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

(1) Cihak, R. ''Evaluation of benzidine by the micronucleus test,'' *Mutation Research,* 67: 383–384 (1979).

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(7) Heddle, J.A., Hite, M., Kurkhart, B., Mavournin, K., MacGregor, J.T., Newell, G.W., Salamone, M.F. ''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).

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§ 798.5450 Rodent dominant lethal assay.

(a) *Purpose.* Dominant lethal (DL) effects cause embryonic or fetal death. Induction of a dominant lethal event after exposure to a chemical substance indicates that the substance has affected germinal tissue of the test species. Dominant lethals are generally accepted to be the result of chromosomal damage (structural and numerical anomalies) but gene mutations and toxic effects cannot be excluded.

(b) *Definition.* A dominant lethal mutation is one occurring in a germ cell which does not cause dysfunction of the gamete, but which is lethal to the fertilized egg or developing embryo.

(c) *Reference substances.* These may include, but need not be limited to, triethylenemelamine, cyclophosphamide or ethyl methanesulfonate.

(d) *Test method*—(1) *Principle.* Generally, male animals are exposed to the test substance and mated to untreated virgin females. The various germ cell stages can be tested separately by the use of sequential mating intervals. The females are sacrificed after an appropriate period of time and the contents of the uteri are examined to determine the numbers of implants and live and dead embryos. The calculation of the dominant lethal effect is based on comparison of the live implants per female in the treated group to the live implants per female in the control group. The increase of dead implants per female in the treated group over the dead implants per female in the control group reflects the post-implantation loss. The post-implantation loss is calculated by determining the ratio of dead to total implants from the treated group compared to the ratio of dead to total implants from the control group. Pre-implantation loss can be estimated on the basis of corpora lutea counts or by comparing the total implants per female in treated and control groups.