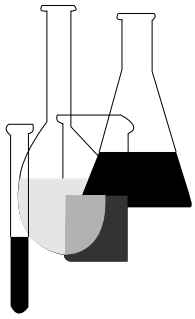
United States
Environmental
Protection Agency

Prevention, Pesticides
and Toxic Substances
(7101)

EPA 712–C–96–226
June 1996

Health Effects Test Guidelines

OPPTS 870.5395 In Vivo Mammalian Cytogenetics Tests: Erythrocyte Micronucleus Assay



“Public Draft”

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.*).

Public Draft Access Information: This draft guideline is part of a series of related harmonized guidelines that need to be considered as a unit. *For copies:* These guidelines are available electronically from the EPA Public Access Gopher (gopher.epa.gov) under the heading ‘‘Environmental Test Methods and Guidelines’’ or in paper by contacting the OPP Public Docket at (703) 305–5805 or by e-mail: guidelines@epamail.epa.gov.

To Submit Comments: Interested persons are invited to submit comments. By mail: Public Docket and Freedom of Information Section, Office of Pesticide Programs, Field Operations Division (7506C), Environmental Protection Agency, 401 M St. SW., Washington, DC 20460. In person: bring to: Rm. 1132, Crystal Mall #2, 1921 Jefferson Davis Highway, Arlington, VA. Comments may also be submitted electronically by sending electronic mail (e-mail) to: guidelines@epamail.epa.gov.

Final Guideline Release: This guideline is available from the U.S. Government Printing Office, Washington, DC 20402 on *The Federal Bulletin Board*. By modem dial 202–512–1387, telnet and ftp: fedbbs.access.gpo.gov (IP 162.140.64.19), or call 202–512–0132 for disks or paper copies. This guideline is also available electronically in ASCII and PDF (portable document format) from the EPA Public Access Gopher (gopher.epa.gov) under the heading ‘‘Environmental Test Methods and Guidelines.’’

OPPTS 870.5395 In vivo mammalian cytogenetics tests: Erythrocyte micronucleus assay.

(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 material used in developing this harmonized OPPTS test guideline is OPPT 40 CFR 798.5395 In Vivo Mammalian Bone Marrow Cytogenetics Tests: Micronucleus Assay; OPP 84–2 Mutagenicity Testing (Pesticide Assessment Guidelines, Subdivision F—Hazard Evaluation; Human and Domestic Animals) EPA report 540/09–82–025, 1982; and OECD 474 Genetic Toxicology: Micronucleus Test.

(b) **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.

(c) **Definition**. The definitions in section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) apply to this test guideline. The following definition also applies to this test guideline.

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.

(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.

(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 h after treatment, with appropriate intervals following the first sample but not extending beyond 72 h. When other doses are used sampling shall be at the maximum sensitive period, or, if that is not known, approximately 24 h 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 h after the last treatment and at appropriate intervals following the first sample, but not extending beyond 72 h.

(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 posi-

tive 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.** The following references should be consulted for additional background material on this test guideline.

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

(2) Cole, R.J. et al. Short-term tests for transplacentally active carcinogens. 1. Micronucleus formation in fetal and maternal mouse erythroblasts. *Mutation Research* 80: 141–157 (1981).

(3) Kliesch, U. et al. Micronucleus test and bone-marrow chromosome analysis. A comparison of 2 methods in vivo for evaluating chemically induced chromosomal alterations. *Mutation Research* 80: 321–332 (1981).

(4) Matter, B. and Schmid, W. Trenimon-induced chromosomal damage in bone-marrow cells of six mammalian species, evaluated by the micronucleus test. *Mutation Research* 12: 417–425 (1971).

(5) Schmid, W. The micronucleus test. *Mutation Research* 31:9–15 (1975).

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

(7) 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).