OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Mammalian Erythrocyte Micronucleus Test

INTRODUCTION

1. The OECD Guidelines for the Testing of Chemicals are periodically reviewed in the light of scientific progress. The original Test Guideline 474 was adopted in 1983. In 1997 a revised version was adopted, based on scientific progress made to that date. Modifications within the current version reflect nearly thirty years of experience with this assay, and in particular the advances in automated scoring technologies and the potential for integration of this test with other studies.

2. The mammalian in vivo micronucleus test is used for the detection of damage induced by the test substance to the chromosomes or the mitotic apparatus of erythroblasts. The test evaluates micronucleus formation in erythrocytes sampled either in the bone marrow or peripheral blood cells of animals, usually rodents.

3. The purpose of the micronucleus test is to identify substances that cause cytogenetic damage that results in the formation of micronuclei containing either lagging chromosome fragments or whole chromosomes.

4. Although not normally done as part of the test, chromosome fragments can be distinguished from whole chromosomes by a number of criteria. These include identification of the presence or absence of a kinetochore or centromeric DNA, both of which are characteristic of intact chromosomes.

5. When a bone marrow erythroblast develops into an immature erythrocyte, the main nucleus is extruded; any micronucleus that has been formed may remain behind in the cytoplasm. Visualisation or detection of micronuclei is facilitated in these cells because they lack a main nucleus. An increase in the frequency of micronucleated immature erythrocytes in treated animals is an indication of induced chromosomal damage.

6. Newly formed micronucleated erythrocytes are identified and quantitated by staining followed by either visual scoring using a microscope, or by automated analysis using image analysis, flow cytometry or laser scanning cytometric analysis. Counting sufficient immature erythrocytes in the peripheral blood or bone marrow of adult animals is greatly facilitated by using image analysis or flow cytometry.

7. Definitions used are set out in the Annex.

INITIAL CONSIDERATIONS

8. The bone marrow of rodents has been used in this test since erythrocytes are produced in that tissue and rodents are widely used in safety testing. The measurement of immature erythrocytes in peripheral blood is equally acceptable in any species in which splenic removal of micronucleated erythrocytes does not significantly interfere with determining the frequency of micronuclei in the cell population used in the study, and for which adequate sensitivity to detect agents that cause structural or numerical chromosomal aberrations has been demonstrated. The frequency of micronucleated immature
erythrocytes is the principal endpoint. The number of mature erythrocytes in the peripheral blood that contain micronuclei among a given number of mature erythrocytes also can be used as an endpoint in species without strong splenic selection against micronucleated cells when animals are treated continuously for 4 weeks or more.

9. The mammalian in vivo micronucleus test is especially relevant for assessing genotoxicity because factors of in vivo metabolism, pharmacokinetics and DNA repair processes are incorporated, although they may vary among species. An in vivo assay is also useful for further investigation of genotoxicity detected by an in vitro system.

10. If there is evidence that the test substance, or its metabolite(s), will not reach the bone marrow, it is not appropriate to use this test.

PRINCIPLE OF THE TEST METHOD

11. Animals are exposed to the test substance by an appropriate route. If bone marrow is used, the animals are sacrificed at an appropriate time(s) after treatment, the bone marrow is extracted, and preparations are made and stained (1)(2)(3)(4)(5)(6)(7). When peripheral blood is used, the blood is collected at an appropriate time(s) after treatment and preparations are made and stained (4)(8)(9)(10). When treatment is administered acutely, it is important to select bone marrow or blood harvest times at which the treatment-related induction of micronucleated newly formed erythrocytes can be detected. In the case of peripheral blood sampling, enough time must also have elapsed for these events to appear in circulating blood. If three or more administrations are performed, it is generally acceptable to collect bone marrow or blood at any time up to 24 hours following the last treatment. Preparations are analyzed for the presence of micronuclei, either by visualization using a microscope, image analysis, flow cytometry, or laser scanning cytometry.

VERIFICATION OF LABORATORY PROFICIENCY

12. Competency in the conduct of these assays should be established by demonstrating experimentally the ability to reproduce expected results (including weak responses) from published data (15)(16)(17) for micronucleus frequencies with positive control substances such as those listed in Table 1, and with vehicle/solvent controls. During the course of these investigations, the laboratory can establish: a historical positive control range and distribution, and a historical negative control range and distribution. Re-evaluation of laboratory proficiency is recommended if major changes to the experimental conditions (eg., new animal strain, new scorer, etc.) are proposed for the assay.

DESCRIPTION OF THE METHOD

Preparations

Selection of animal species

13. Commonly used laboratory strains of healthy young adult animals should be employed. Mice, rats, or another appropriate mammalian species may be used. When peripheral blood is used, it must be established that splenic removal of micronucleated cells from the circulation does not compromise the detection of induced micronuclei in the species selected. At the commencement of the study, the weight variation of animals should be minimal and not exceed ± 20% of the mean weight of each sex. While the majority of erythrocyte-based micronucleus tests are conducted with laboratory rodents, there has been growing experience and effective use of the endpoint in other species, including canine, non-human primate, and human species (for example, (11)(12)(13)(14)).
Animal housing and feeding conditions

14. For rodents, the temperature in the animal room should be 22°C (±3°C). Although the relative humidity ideally should be 50-60%, it should be at least 30% and preferably not exceed 70% other than during room cleaning. 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 route. Rodents should be housed in small groups (no more than five) of the same sex if no aggressive behaviour is expected. Animals may be housed individually only if scientifically justified.

Preparation of the animals

15. Healthy young adult animals (for rodents, 6-10 weeks old at start of treatment) are normally used, and are randomly assigned to the control and treatment groups. The animals are identified uniquely and acclimated to the laboratory conditions for at least five days. Cages should be arranged in such a way that possible effects due to cage placement are minimized. At the commencement of the study, the weight variation of animals should be minimal and not exceed ± 20% of the mean weight of each sex.

Preparation of doses

16. Solid test substances should be dissolved or suspended in appropriate solvents or vehicles or admixed in diet or drinking water prior to dosing the animals. Liquid test substances may be dosed directly or diluted prior to dosing. For inhalation exposures, test substances can be administered as a gas, vapour, or a solid/liquid aerosol, depending on their physicochemical properties. Fresh preparations of the test substance should be employed unless stability data demonstrate the acceptability of storage.

Test conditions

Solvent/vehicle

17. The solvent/vehicle should not produce toxic effects at the dose levels used, and should not be capable of chemical reaction with the test substance. If other than well-known solvents/vehicles are used, their inclusion should be supported with reference data indicating their compatibility. It is recommended that wherever possible, the use of an aqueous solvent/vehicle should be considered first.

Controls

Positive controls

18. A group of animals treated with a positive control substance should normally be included with each test. This may be waived when the testing laboratory has demonstrated proficiency in the conduct of the test. When a concurrent positive control group is not included, scoring controls should be included in each experiment. These can be obtained by including within the scoring of the study appropriate reference samples that have been obtained and stored from a separate positive control experiment conducted periodically in the laboratory where the test is conducted, for example, during proficiency testing.

19. Positive control compounds should reliably produce a detectable increase in micronucleus frequency over background. Positive control doses should be chosen so that the effects are clear but do not immediately reveal the identity of the coded samples to the scorer, except when using automated scoring systems, which do not rely on visual inspection and cannot be affected by operator bias. It is acceptable that the positive control be administered by a route different from the test substance, using a different treatment schedule, and for sampling to occur only at a single time point. In addition, the use of chemical class-related positive control chemicals may be considered, when available. Examples of positive control substances are included in Table 1.
Table 1. Examples of positive control substances.

<table>
<thead>
<tr>
<th>Chemical and CAS No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl methanesulphonate [CAS no. 62-50-0]</td>
</tr>
<tr>
<td>Ethyl nitrosourea [CAS no. 759-73-9]</td>
</tr>
<tr>
<td>Mitomycin C [CAS no. 50-07-7]</td>
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<tr>
<td>Cyclophosphamide (monohydrate) [CAS no. 50-18-0 (CAS no. 6055-19-2)]</td>
</tr>
<tr>
<td>Triethylenemelamine [CAS no. 51-18-3]</td>
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</table>

Negative controls

20. Negative control animals, treated with solvent or vehicle alone and otherwise handled in the same way as the treatment groups, should be included for each sex used and at every sampling time. However, if consistent inter-animal variability and frequencies of cells with micronuclei are demonstrated by historical negative control data for the testing laboratory, only a single sampling is necessary. Where a single sampling is used for negative controls, it should be the first sampling time used in the study. In the absence of historical or published control data showing that no micronuclei or other deleterious effects are induced by the chosen solvent/vehicle, untreated controls also should be included for every sampling time in order to establish acceptability of the vehicle control.

21. If peripheral blood is used, a pre-treatment sample may also be acceptable as a concurrent negative control, but only in acute-dose peripheral blood studies (e.g., 1-3 treatment(s)) and when the resulting data are in the expected range for the historical control.

PROCEDURE

Number and sex of animals

22. Group sizes will consist of a minimum of 5 analysable animals. In general, only one sex is necessary. However, if there is evidence indicating a relevant difference in toxicity (for example, where clinical observations, bodyweights and/or pathology indicate one sex is more susceptible) or metabolism between males and females, then both should be used. 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.

Treatment schedule

23. Single treatments can be performed; alternatively, 2 or more treatments can be administered at 24-hour intervals. The samples from extended dose regimens (e.g., 28-day daily dosing) are acceptable as long as a positive effect has been demonstrated for this study or, for a negative study, as long as toxicity to the erythropoietic system has been demonstrated or the limit dose has been used, and dosing continued until the time of sampling. Test substances also may 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.

24. The test may be performed in mice or rats in three ways:
a. Animals are treated with the test substance once. Samples of bone marrow are taken at least twice, starting not earlier than 24 hours after treatment, but not extending beyond 48 hours after treatment with appropriate interval(s) between samples, unless the test substance is known to have an exceptionally long half-life. The use of sampling times earlier than 24 hours after treatment should be justified. Samples of peripheral blood are taken at least twice, starting not earlier than 36 hours after treatment, with appropriate intervals following the first sample, but not extending beyond 72 hours. When a positive response is detected at one sampling time, additional sampling is not required unless quantitative dose-response information is needed.

b. If 2 daily treatments are used (e.g. two treatments at 24 hour intervals), samples should be collected once between 18 and 24 hours following the final treatment for the bone marrow and once between 24 and 48 hours following the final treatment for peripheral blood (18).

c. If 3 or more daily treatments are used (e.g. three or more treatments at 24 hour intervals), bone marrow samples should be collected no later than 24 hours after the last treatment. Peripheral blood should be collected no later than 40 hours after the last treatment; however, approximately 24 hours after the last treatment is most practical (18).

25. Other sampling times may be used when relevant and justified scientifically.

Dose levels

26. If a preliminary range-finding study is performed because there are no suitable data available to aid in dose selection, it should be performed in the same laboratory, using the same species, strain, sex, and treatment regimen to be used in the main study (19). The range-finding study should be started with the most likely dose to cause toxicity, using a small number of animals (e.g. 2 per sex). If the maximum tolerated dose (MTD) is not identified, a further group of animals should be exposed to a higher or lower dose depending on the clinical effects of the first dose. This strategy should be repeated until the appropriate MTD is found. The highest dose level should be chosen with the aim of inducing slight toxic effects but not death or evidence of pain, suffering or distress necessitating humane euthanasia.

27. The highest dose may also be defined as a dose that produces toxicity in the bone marrow (e.g. a reduction in the proportion of immature erythrocytes among total erythrocytes in the bone marrow or peripheral blood to 50-20% of the control value). However, when analyzing CD71-positive cells in peripheral blood circulation (i.e., by flow cytometry), this very young fraction of immature erythrocytes responds to toxic challenges more quickly than the larger RNA-positive cohort of immature erythrocytes. Therefore, higher apparent toxicity may be evident with acute exposure designs examining the CD71-positive immature erythrocyte fraction as compared to those that identify immature erythrocytes based on RNA content. For this reason, when experiments utilize five or fewer days of treatment, the highest dose level may be defined as the dose that causes the proportion of peripheral blood CD71-positive immature erythrocytes compared among total erythrocytes to be reduced to approximately 5 - 10% of the control value.

28. Substances with specific biological activities at low non-toxic doses (such as hormones and mitogens), and substances that exhibit saturation of toxicokinetic properties may be exceptions to the dose-setting criteria and should be evaluated on a case-by-case basis.

29. If the test substance produces toxicity, the MTD and a descending sequence of two additional dose levels should be selected for the first sampling time (or only sampling time, as appropriate), with a view to demonstrating any dose-related response. Studies intending to more fully characterize the quantitative dose-response information may require additional dose groups.

Limit test
30. If dose range-finding experiments, or existing data from related animal strains, indicate that a treatment regime of at least the limit dose (described below) produces no observable toxic effects (including no depression of bone marrow proliferation), and if genotoxicity would not be expected based upon in vitro genotoxicity studies or data from structurally related substances, then a full study using three dose levels may not be considered necessary. In such cases, a single dose level, at the limit dose, may be sufficient. For an administration period of 14 days or more, the limit dose is 1000 mg/kg body weight/day. For administration periods of less than 14 days, the limit dose is 2000 mg/kg body weight/day.

Administration of doses

31. In general, the anticipated route of human exposure should be considered when selecting the route of administration. Usually, the test substance is administered by gavage using a stomach tube or a suitable intubation cannula. Other routes of exposure (such as drinking water, subcutaneous, intravenous, topical, inhalation, intratracheal, dietary, or implantation) may be acceptable when relevant to the expected route of human exposure. Intraperitoneal injection is not normally recommended since it is not usually a physiologically relevant route of human exposure. 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 greater than this should be justified. Except for irritating or corrosive substances, which will normally produce exacerbated effects at higher concentrations, variability in test volume should be minimised by adjusting the concentration to ensure administration of a constant volume at all dose levels.

Bone marrow / blood preparation

32. Bone marrow cells are usually obtained from the femurs or tibias of the test animals immediately following euthanasia. Commonly, cells are removed, prepared and stained using established methods. Small volumes of peripheral blood can be obtained either using a method that permits survival of the test animal, such as bleeding from the tail vein or other appropriate blood vessel, or by cardiac puncture or sampling from a large vessel at animal sacrifice. Depending on the method of analysis, blood cells may be immediately stained supravitally (8)(9)(10), smear preparations are made and then stained for microscopy, or fixed and stained appropriately for flow cytometric analysis. The use of a DNA specific stain [e.g. acridine orange (20) or Hoechst 33258 plus pyronin-Y (21)] can eliminate some of the artifacts associated with using a non-DNA specific stain. This advantage does not preclude the use of conventional stains (e.g., Giemsa for microscopic analysis). Additional systems [e.g. cellulose columns to remove nucleated cells (22)] also can be used provided that these systems have been demonstrated to be compatible with sample preparation in the laboratory.

33. Anti-kinetochore antibodies, FISH with pancentromeric DNA probes, or primed in situ labelling with pancentromere-specific primers, together with appropriate DNA counterstaining, can be used to identify the contents (chromosome/chromosomal fragment) of micronuclei in order to determine whether the mechanism of micronucleus induction is due to clastogenic and/or aneugenic activity. Other methods for differentiation between clastogens and aneugens may be used if they have been shown to be effective.

Analysis

34. All slides or samples for analysis, including those of positive and negative controls, should be independently coded before any type of analysis and should be randomized so the scorer is unaware of the treatment condition. The proportion of immature among total (immature + mature) erythrocytes is determined for each animal by counting a total of at least 200 erythrocytes for bone marrow and 1000 erythrocytes for peripheral blood (23). [Considerations of statistical power and the number of cells to be scored were removed from the current draft, as they are topics that will be discussed at an upcoming expert group meeting]. Additional information may be obtained by scoring mature erythrocytes for micronuclei. When analysing samples, the proportion of immature erythrocytes among total erythrocytes in treated animals should not be less than 20% of the vehicle/solvent control value when scoring by
microscopy and not less than approximately 5% of the vehicle/solvent control value when scoring CD71+

35. Because the rat spleen sequesters and destroys micronucleated erythrocytes, to maintain high
assay sensitivity when analysing rat peripheral blood, it is preferable to restrict the analysis of
micronucleated immature erythrocytes to the youngest fraction. These most immature erythrocytes can be
identified based on their high RNA content, or the high level of transferrin receptors expressed on their
surface (24). However, direct comparison of different staining methods has shown that satisfactory
results can be obtained with various methods, including conventional acridine orange staining (26)(27).

Automated Assays

36. Systems for automated analysis are acceptable alternatives to manual evaluation (28). Comparative
studies have shown that such methods, using appropriate calibration standards, can provide
better inter- and intra-laboratory reproducibility and sensitivity than manual microscopic scoring (26)(27).

37. Automated systems that can measure micronucleated erythrocyte frequencies include, but are not
limited to, flow cytometers, image analysis platforms, and laser scanning cytometers.

Observations

38. General clinical observations of the test animals should be made at least once a day, preferably at
the same time(s) each day and considering the peak period of anticipated effects after dosing. The health
condition of the animals should be recorded. At least twice daily during the dosing period, all animals
should be observed for morbidity and mortality. All animals should be weighed at least once a week, and at
sacrifice. Measurements of food consumption should be made at least weekly. If the test substance is
administered via the drinking water, water consumption should be measured at each change of water and
at least weekly. Animals exhibiting non-lethal indicators of excessive toxicity should be euthanized prior to
completion of the test period (29). Under certain circumstances, animal body temperature could be
monitored, since treatment-induced hyper- and hypothermia have been implicated in producing spurious
results (30)(31)(32).

DATA AND REPORTING

Treatment of Results

39. Individual animal data should be presented in tabular form. The number of immature erythrocytes scored,
the number of micronucleated immature erythrocytes, and the number of immature among total erythrocytes
should be listed separately for each animal analysed. When mice are treated continuously for 4 weeks or more,
the data on mature erythrocytes also should be given if collected. The proportion of immature among total
erythrocytes and, if considered applicable, the percentage of micronucleated erythrocytes is given for each animal.
[Considerations of statistical power and the number of cells to be scored were removed from the current
draft, as they are topics that will be discussed at an upcoming expert group meeting]. Data on animal
toxicity and clinical signs should also be reported.

Evaluation and Interpretation of Results

40. The biological relevance of any increases in micronucleus frequency should be the primary
consideration in deciding whether or not a test substance is positive. However, appropriate statistical
methods should first be used in evaluating the test results (33)(34)(35)(36). There are several additional
criteria that are useful for determining a positive result, such as a dose-related increase in the
micronucleus frequency, or a clear increase in the micronucleus frequency in a single dose group
compared to the solvent/vehicle control group. When conducting a dose-response analysis, at least three
treated dose groups should be analysed. Statistical tests should use the animal as the experimental unit.
41. Consideration of whether the observed values are within or outside of the historical control range can provide guidance when evaluating the biological significance of the response (14)(15)(16).

42. The likelihood that the test substance or its metabolites reach the general circulation (e.g. systemic toxicity) or the bone marrow should be discussed.

43. A test substance for which the results do not meet the above criteria for a positive result is considered negative in this assay if all criteria for a valid assay are met; otherwise the result is considered equivocal. To establish the biological relevance of a negative result, bone marrow exposure should be confirmed, such as by verifying clinical signs of systemic toxicity, measurement of the immature to mature erythrocyte ratio or by measuring plasma levels of the test substance. Equivocal results should be clarified by further testing preferably using a modification of experimental conditions.

44. Positive results in the micronucleus test indicate that a substance induces micronuclei, which are the result of chromosomal damage or damage to the mitotic apparatus in the erythroblasts of the test species. A test substance that produces centromere or kinetochore-containing micronuclei (indicative of whole chromosome loss) is evidence the test substance is an aneugen. Negative results indicate that, under the test conditions, the test substance does not produce micronuclei in the immature erythrocytes of the test species.

**Test Report**

45. The test report should also include the following information:

*Test substance:*

- identification data and CAS number;
- source, lot number if available;
- physical nature and purity;
- physicochemical properties relevant to the conduct of the study;
- stability of the test substance, if known.

*Solvent/vehicle:*

- justification for choice of vehicle;
- solubility and stability of the test substance in the solvent/vehicle, if known;
- preparation of dietary, drinking water or inhalation formulations;
- analytical determinations on formulations (e.g., stability, homogeneity, nominal concentrations).

*Test animals:*

- species/strain used;
- number, age and sex of animals;
- source, housing conditions, diet, etc.;
- individual weight of the animals at the start of the test, including body weight range, mean and standard deviation for each group.

*Test conditions:*

- positive and negative (vehicle/solvent) control data;
- data from range-finding study, if conducted;
- rationale for dose level selection;
- details of test substance preparation;
- details of the administration of the test substance;
- rationale for route and duration of administration;
- methods for verifying that the test substance reached the general circulation or target tissue, if applicable;
- actual dose (mg/kg body weight/day) calculated from diet/drinking water test substance concentration (ppm) and consumption, if applicable;
- details of food and water quality;
- method of euthanasia;
- detailed description of treatment and sampling schedules and justifications for the choices;
- methods of slide preparation;
- procedures for isolating and preserving samples;
- methods for measurement of toxicity;
- criteria for scoring micronucleated immature erythrocytes;
- number of cells analysed per animal;
- criteria for considering studies as positive, negative or equivocal;
- methods, such as use of anti-kinetochore antibodies, to characterise whether micronuclei contain whole or fragmented chromosomes, if applicable.

Results:

- animal condition prior to and throughout the test period, including signs of toxicity;
- proportion of immature erythrocytes among total erythrocytes;
- number of micronucleated immature erythrocytes, given separately for each animal;
- mean ± standard deviation of micronucleated immature erythrocytes per group;
- dose-response relationship, where possible;
- statistical analyses and method applied;
- concurrent and historical negative control data with ranges, means and standard deviations;
- positive or scoring control data;
- analytical determinations for exposure of bone marrow, if applicable;
- characterisation data indicating whether micronuclei contain whole or fragmented chromosomes, if applicable

Discussion of the results.

Conclusion.


ANNEX

DEFINITIONS

Centromere: Region(s) of a chromosome with which spindle fibers are associated during cell division, allowing orderly movement of daughter chromosomes to the poles of the daughter cells.

Micronuclei: small nuclei, separate from and additional to the main nuclei of cells, produced during telophase of mitosis (meiosis) by lagging chromosome fragments or whole chromosomes.

Mature erythrocyte: fully developed erythrocyte that lacks ribosomes and can be distinguished from immature erythrocytes by stains selective for ribosomes.

Immature erythrocyte: newly-formed erythrocyte, in an intermediate stage of development, that still contains ribosomes and therefore can be distinguished from mature erythrocytes by stains selective for ribosomes, or by using certain antibody-fluorochrome complexes. Polychromatic erythrocytes, reticulocytes, and CD71-positive erythrocytes are all immature erythrocytes, though each has a somewhat different age distribution.

Polychromatic erythrocyte: a newly formed erythrocyte that stains with both the blue and red components of classical blood stains such as Wright's Giemsa because of the presence of residual RNA in the newly-formed cell. Such newly formed cells are approximately the same as reticulocytes, which are visualized using a vital stain that causes the residual RNA to clump into a reticulum. Other methods, including monochromatic staining of RNA with fluorescent dyes or labeling of short-lived surface markers such as CD71 with fluorescent antibodies, are now often used to identify the newly formed red blood cell.

Reticulocyte: A newly formed erythrocyte stained with a vital stain that causes residual cellular RNA to clump into a characteristic reticulum. Reticulocytes and polychromatic erythrocytes have a similar cellular age distribution.

Normochromatic erythrocyte: A fully matured erythrocyte that has lost the residual RNA that remains after enucleation and/or has lost other short-lived cell markers that are characteristically lost after enucleation following the final erythroblast division.