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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, changing regulatory needs and animal welfare considerations. 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 integrating or combining this test with other general toxicity or genotoxicity studies.

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

3. The mammalian *in vivo* micronucleus test is used for the detection of damage induced by the test chemical 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.

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

5. When a bone marrow erythroblast develops into an immature erythrocyte (sometimes also referred to as a polychromatic erythrocyte or reticulocyte), 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. 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. The absence of kinetochore or centromeric DNA indicates that the micronucleus contains only fragments of chromosomes, while the presence is indicative of chromosome loss.

8. Definitions of terminology used are set out in Annex 1.

INITIAL CONSIDERATIONS

9. The bone marrow of young adult rodents is the target tissue for genetic damage in this test since erythrocytes are produced in this tissue and rodents are widely used in safety testing. The measurement of immature erythrocytes in peripheral blood is equally acceptable in any mammalian species 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 frequency of mature erythrocytes that contain micronuclei in the peripheral blood also can be used as an endpoint in species without strong splenic selection against micronucleated cells and when animals are treated continuously for 4 weeks or more.

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

PRINCIPLE OF THE TEST METHOD

11. Animals are exposed to the test chemical by an appropriate route. If bone marrow is used, the animals are humanely euthanized 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. 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

Proficiency Investigations

12. In order to establish sufficient experience with the conduct of the assay prior to using it for routine testing, the laboratory should have demonstrated experimentally, within the tissue of interest (bone marrow or peripheral blood) and using the scoring method to be employed within the laboratory, the ability to reproduce expected results from published data (11)(12)(13) for micronucleus frequencies with a minimum of two positive control chemicals (including weak responses induced by low doses of positive controls), such as those listed in Table 1 and with compatible vehicle/solvent controls (see Paragraph 22).

Historical Control Data

13. During the course of the proficiency investigations, the laboratory should establish:

- A historical positive control range and distribution, and
- A historical negative control range and distribution.

14. Laboratories should use quality control methods, such as control charts (e.g. C-charts or X-bar charts (14)), to identify how variable their data are, and to show that the methodology is 'under control' in their laboratory (see Annex 2). Negative control data should consist of the incidence of micronucleated immature erythrocytes in each animal within a group of five analysable animals. Concurrent negative controls should ideally be within the Poisson-based 99% control limits of the distribution of the laboratory's historical control database measured in at least 10 experiments conducted under the same experimental conditions. In those rare situations (around 1%) where data fall close to the existing historical control range and for which there is no evidence that the test system has gone out of control and no evidence of

technical or human failure, the data should be acceptable for evaluation and acceptable for addition to the historical database. Other recommendations on how to build and use the historical data (i.e. criteria for inclusion and exclusion of data in historical data and the acceptability criteria for a given experiment) can be found in the literature (15). Renewal/re-establishment of historical ranges is recommended if major changes to the experimental conditions (e.g., new animal strain, new scoring method, etc.) are proposed for the assay.

DESCRIPTION OF THE METHOD

Preparations

Selection of animal species

15. 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. This has been clearly demonstrated for mouse and rat peripheral blood (16). The scientific justification for using species other than rats and mice should be provided in the report. If species other than rodents are used, it is recommended that the measurement of induced micronuclei be integrated into another appropriate toxicity test.

Animal housing and feeding conditions

16. 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 chemical when administered by this route. Rodents should be housed in small groups (no more than five per cage) of the same sex if no aggressive behaviour is expected, preferably in solid floor cages with appropriate environmental enrichment. Animals may be housed individually only if scientifically justified.

Preparation of the animals

17. 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 individual 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. Cross contamination by the positive control and the test chemical should be minimized. At the commencement of the study, the variation between individual animal weights and the mean weight of each corresponding sex group should be minimal and not exceed ± 20%.

Preparation of doses

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

Test Conditions

Solvent/vehicle

19. The solvent/vehicle should not produce toxic effects at the dose levels used, and should not be capable of chemical reaction with the test chemical. 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

20. A group of animals treated with a positive control chemical should normally be included with each test. This may be waived when the testing laboratory has demonstrated proficiency in the conduct of the test and has established a historical positive control range. When a concurrent positive control group is not included, scoring controls (fixed and unstained slides or cell samples) 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 (e.g. every 12-18 months) in the laboratory where the test is performed; for example, during proficiency testing and on a regular basis thereafter, where necessary.

21. Positive control compounds should reliably produce a detectable increase in micronucleus frequency over the spontaneous level. 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 chemical, 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 appropriate. Examples of positive control chemicals are included in Table 1.

Table 1. Examples of positive control chemicals.

Chemical and CAS No.
Ethyl methanesulphonate [CAS no. 62-50-0]
Methyl methanesulphonate [CAS no. 66-27-3]
Ethyl nitrosourea [CAS no. 759-73-9]
Mitomycin C [CAS no. 50-07-7]
Cyclophosphamide (monohydrate) [CAS no. 50-18-0 (CAS no. 6055-19-2)]
Triethylenemelamine [CAS no. 51-18-3]
Colchicine [CAS no. 64-86-8] or Vinblastine [CAS no. 865-21-4] – as aneugens

Negative controls

22. Negative control group animals should be included for each sex used and at every sampling time and otherwise handled in the same way as the treatment groups, except for not receiving treatment

with the test chemical. If a solvent/vehicle is used in administering the test chemical, the control group should receive the solvent/vehicle. 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 for the negative control is necessary. Where a single sampling is used for negative controls, it should be the first sampling time used in the study. Examples of commonly used compatible solvents/vehicles include water, physiological saline, methylcellulose solution, carboxymethyl cellulose sodium salt solution, olive oil and corn oil. In the absence of historical or published control data showing that no micronuclei or other deleterious effects are induced by a chosen atypical solvent/vehicle, untreated controls also should be included for every sampling time in order to establish the acceptability of the solvent/vehicle control.

23. If peripheral blood is used, a pre-treatment sample is acceptable as a concurrent negative control when the resulting data are consistent with the historical control database for the testing laboratory.

PROCEDURE

Number and sex of animals

24. Group sizes at study initiation should be established with the aim of providing a minimum of 5 analysable animals per group. In general, only one sex is necessary. However, if there is evidence indicating a relevant difference in toxicity (for example, where clinical observations, body weights and/or pathology indicate one sex is more susceptible) or metabolism between males and females, then either both sexes or the most relevant/susceptible sex 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 the appropriate sex. As a guide to maximum typical animal requirements, a study in bone marrow conducted according the parameters established in Paragraph 31 with three dose groups and concurrent negative and positive controls (each group composed of five animals of a single sex) would require between 25 and 35 animals.

Dose levels

25. If a preliminary range-finding study is performed because there are no suitable data already 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 (17), according to current approaches for conducting dose range-finding studies. The study should aim to identify the maximum tolerated dose (MTD), defined as the dose inducing slight toxic effects relative to the duration of the study period (for example, abnormal behaviour or reactions, minor body weight depression or hematopoietic system cytotoxicity), but not death or evidence of pain, suffering or distress necessitating humane euthanasia (18).

26. 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 of more than 50%, but to not less than 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 for test chemicals causing toxicity 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 not less than 5% of the control value (19).

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

28. In the main study, in order to obtain dose response information, a complete study should include a negative control group and a minimum of three dose levels generally separated by a factor of 2, but by no greater than 4. If the test chemical does not produce toxicity in a range-finding study or based on existing data, the highest dose for an administration period of 14 days or more should be 1000 mg/kg body weight/day, or for administration periods of less than 14 days, 2000 mg/kg/body weight/day. However, if the test chemical does cause toxicity, the MTD should be the highest dose administered. Studies intending to more fully characterize the quantitative dose-response information may require additional dose groups.

Administration of doses

29. In general, the anticipated route of human exposure should be considered when selecting the route of administration. Usually, the test chemical 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 it leads to an exposure relevant to the expected route of human exposure. If the test chemical is admixed in diet or drinking water, especially in case of single dosing, care should be taken that the delay between food and water consumption and sampling should be sufficient to allow detection of the effects (see Paragraph 31). Intraperitoneal injection is not normally recommended unless scientifically justified, 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 normally exceed 1 mL/100 g body weight except in the case of aqueous solutions where a maximum of 2 mL/100 g may be used. The use of volumes greater than this should be justified. Except for irritating or corrosive test chemicals, 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.

Treatment schedule

30. Single treatments can be performed; alternatively, 2 or more treatments can be administered at 24-hour intervals. Test chemicals also may be administered as a split dose, i.e., two treatments on the same day separated by no more than 2-3 hours, to facilitate administering a large volume. Under these circumstances, the sampling time should be scheduled based on the time of the last dosing.

31. The test may be performed in mice or rats in two ways:

a. Animals are treated with the test chemical once. Samples of bone marrow are taken at least twice (from independent groups of animals), 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 chemical 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 (from the same group of animals), starting not earlier than 36 hours after treatment, with appropriate intervals following the first sample, but not extending beyond 72 hours. At the first sampling time, all dose groups should be treated and samples collected for analysis; however, at the later sampling time(s), only the highest dose needs to be administered. 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 or more daily treatments are used (e.g. two or more 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 40 hours following the final treatment for peripheral blood (20).

32. When a combined micronucleus test and comet assay is performed, animals should be treated at three intervals of 0, 24 and 45 hours and bone marrow sampled at 48 hours (3 hours after the last dose) in

order to accommodate the sampling requirements of the comet assay (21). Other dosing or sampling regimens may be used when relevant and scientifically justified.

Observations

33. General clinical observations of the test animals should be made and clinical signs recorded at least once a day, preferably at the same time(s) each day and considering the peak period of anticipated effects after dosing. 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 during repeated dose studies, and at sacrifice. Measurements of food consumption should be made at least weekly. If the test chemical 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 humanely euthanized prior to completion of the test period (18). Under certain circumstances, animal body temperature could be monitored, since treatment-induced hyper- and hypothermia have been implicated in producing spurious results (22)(23)(24).

Bone marrow / blood preparation

34. Bone marrow cells are usually obtained from the femurs or tibias of the animals immediately following humane euthanasia. Commonly, cells are removed, prepared and stained using established methods. Small volumes of peripheral blood can be obtained, according to adequate animal welfare standards, 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. For both bone marrow or peripheral blood-derived erythrocytes, depending on the method of analysis, 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 (25) or Hoechst 33258 plus pyronin-Y (26)] 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 (27)] also can be used provided that these systems have been demonstrated to be compatible with sample preparation in the laboratory.

35. Where these methods are applicable, anti-kinetochore antibodies (28), FISH with pancentromeric DNA probes (29), or primed *in situ* labelling with pancentromere-specific primers, together with appropriate DNA counterstaining (30), can be used to identify the nature of the micronuclei (chromosome/chromosomal fragment) 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

36. 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 500 erythrocytes for bone marrow and 2000 erythrocytes for peripheral blood (31). At least 5000 immature erythrocytes per animal should be scored for the incidence of micronucleated immature erythrocytes. However, if the historical negative control database indicates the mean background micronucleated immature erythrocyte frequency is <0.1% in the testing laboratory, consideration should be given to scoring additional cells. 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+ immature erythrocytes by cytometric methods (see Paragraph 26) (19).

37. 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. When using automated analysis methods, these most immature erythrocytes can be identified based on their high RNA content, or the high level of transferrin receptors (CD71+) expressed on their surface (32). However, direct comparison of different staining methods has shown that satisfactory results can be obtained with various methods, including conventional acridine orange staining (33)(34).

Automated Assays

38. Systems for automated analysis are acceptable alternatives to manual evaluation (35). 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 (33)(34).

39. Automated systems that can measure micronucleated erythrocyte frequencies include, but are not limited to, flow cytometers (36), image analysis platforms (37)(38), and laser scanning cytometers (39).

DATA AND REPORTING

Treatment of Results

40. Individual animal data should be presented in tabular form. The number of immature erythrocytes scored, the number of micronucleated immature erythrocytes, and the proportion 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. Data on animal toxicity and clinical signs should also be reported.

Acceptability Criteria

41. The following criteria determine the acceptability of the test:

a) The concurrent negative control data are consistent with the historical negative control data in the testing laboratory (see Paragraph 14).

b) The concurrent positive controls or scoring controls fulfil the positivity criteria (see Paragraph 20-21).

c) The appropriate number of doses and cells has been analysed.

d) The criteria for the selection of highest dose are consistent with those described in Paragraphs 25-28.

Evaluation and Interpretation of Results

42. Providing that all acceptability criteria are fulfilled, a test chemical is considered clearly positive if at least one of the doses exhibits a statistically significant increase in the frequency of micronucleated immature erythrocytes compared with the concurrent negative control and the increase is dose-related at least at one sampling time. If only the highest dose is examined at a particular sampling time, a test chemical is considered clearly positive if there is a statistically significant increase compared with the concurrent negative control. Recommendations for the most appropriate statistical methods can be found in the literature (40)(41)(42)(43). 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. Positive results in the micronucleus test indicate that a test chemical induces micronuclei, which are the result of chromosomal damage or damage to the mitotic apparatus in the erythroblasts of the test species. In the

case where a test was performed to detect centromeres within micronuclei, a test chemical that produces centromere-containing micronuclei (centromeric DNA or kinetochore, indicative of whole chromosome loss) is evidence the test chemical is an aneugen.

43. Providing that all acceptability criteria are fulfilled, a test chemical is considered clearly negative if none of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control, there is no dose-related increase at any sampling time, and bone marrow exposure to the test chemical occurred. Recommendations for the most appropriate statistical methods can be found in the literature (40)(41)(42)(43). A depression of the immature to mature erythrocyte ratio or measurement of the plasma or blood levels of the test chemical (except when the intravenous route of exposure is chosen) can establish that bone marrow exposure to the test chemical occurred. Alternatively, ADME data, obtained in an independent study using the same route and same species can be used, if the dose is not below half of the highest dose used in the micronucleus assay. Negative results indicate that, under the test conditions, the test chemical does not produce micronuclei in the immature erythrocytes of the test species.

44. There is no requirement for verification of a clear positive or clear negative response.

45. In cases where the response is not clearly negative or positive and in order to assist in establishing the biological relevance of a result (e.g. a weak or borderline increase), the data should be evaluated by expert judgement and/or further investigations of the existing experiments completed, such as determining if the positive result is outside the distribution of the historical negative control data (e.g. Poisson-based 95% control limit; see Paragraph 14 and Annex 2). In some cases, analysing more cells or performing a repeat experiment using slightly modified experimental conditions could be useful.

46. In rare cases, even after further investigations, the data will preclude making a conclusion that the test chemical produces either positive or negative results, and the study will therefore be concluded as inconclusive.

Test Report

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

Summary

Test chemical:

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

Solvent/vehicle:

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

Test animals:

- species/strain used and justification for use;
- number, age and sex of animals;
- source, housing conditions, diet, etc.;

- individual weight of the animals at the start and end 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 chemical preparation;
- details of the administration of the test chemical;
- rationale for route and duration of administration;
- methods for verifying that the test chemical reached the general circulation or target tissue;
- actual dose (mg/kg body weight/day) calculated from diet/drinking water test chemical 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 in determining the frequency of micronucleated immature erythrocytes and for determining the proportion of immature to mature erythrocytes;
- criteria for acceptability of the study;
- methods, such as use of anti-kinetochore antibodies or centromere-specific DNA probes, 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 negative and positive (or scoring) control data with ranges, means and standard deviations;
- historical negative and positive control data with ranges, means, standard deviations and 95% confidence interval, as well as the time period covered and the number of data points;
- data providing evidence of bone marrow exposure;
- characterisation data indicating whether micronuclei contain whole or fragmented chromosomes, if applicable

Discussion of the results.

Conclusion.

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ANNEX 1

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.

Erythroblast: An early stage of erythrocyte development, immediately preceding the immature erythrocyte, where the cell still contains a nucleus.

Kinetochore: The protein structure that forms on the centromere of eukaryotic cells, which links the chromosome to microtubule polymers from the mitotic spindle during mitosis and meiosis and functions during cell division to pull sister chromatids apart.

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.

Normochromatic or mature 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 characteristically disappear after enucleation following the final erythroblast division.

Polychromatic or immature erythrocyte: A newly formed erythrocyte in an intermediate stage of development, 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. Polychromatic erythrocytes, reticulocytes, and CD71-positive erythrocytes are all immature erythrocytes, though each has a somewhat different age distribution.

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.

ANNEX 2

STATISTICAL CONSIDERATIONS ON THE USE OF HISTORICAL DATA

Historical negative control data are used both to assist in determining the acceptability of the assay, and to help with the interpretation of the results in those cases where results are neither clearly negative nor clearly positive. Quality control methods (for example C-charts) should be used to characterise the distribution of the negative control database.

These methods can be used to assess the acceptability of the assay, using the 99% control limits. In order to avoid narrowing the database, all data should be included in the historical database, even when rejected for the evaluation of the results, unless a technical or human failure has been identified. In this case the data will not be added to the database.

In the interpretation of the results, the treated values should be compared with the 95% control levels for the distribution of the historical negative control database. Results that fall within the 95% control levels for the historical control could be considered broadly consistent with historical negative control data. The limit of 95% is a more conservative approach than the 99% value used for acceptability criteria, and is considered adequate for the evaluation of results.

ANNEX 3

STATISTICAL CONSIDERATIONS ON THE NUMBER OF CELLS TO SCORE

Micronucleated immature erythrocytes (MN-IEs) arise spontaneously at low frequencies, typically from 0.02 to 0.2% ([1][2]; unpublished survey), which has focused attention on the question of whether maintaining the recommendation to score 2000 IEs per animal will provide sufficient statistical power to detect treatment-induced increases in the MN-IE frequency. Historically, counting this number of cells was based, to a large degree, on the number that could reasonably be scored by a microscopist.

Recommendations on the number of IEs to score have been made as an outcome of several past International Workshops on Genotoxicity Testing (IWGT). The 2nd IWGT expert meeting of March 25-26, 1999 recommended that, "Sufficient cells should be analyzed to assure the detection of a doubling in micronucleus frequency with 80% power at a significance level of less than 0.05" [3]. Subsequently, the 4th IWGT meeting of September 8-9, 2005 further recommended that, "...it is desirable that each laboratory should determine the minimum cell sample size required to ensure that scoring error is maintained below the level of animal-to-animal variation" [4].

The power of various experimental designs has been considered in several studies [5][6], which have indicated scoring 2000 IEs does not provide sufficient power to detect a doubling in MN-IE frequency with 80% power at a significance level of less than 0.05. In general, studies employing sample sizes of 5 animals/group and scoring 4000-5000 cells per animal will have approximately 80% power to detect about 2-3 fold increases when the spontaneous frequency is ≥0.1%, but that the power will be much smaller for lower background levels.

Consequently, scoring 5000 cells per animal would represent a compromise between achieving the most robust statistical power of the assay and the practicability of scoring by microscopy. This number of cells also takes into account that where Poisson distributed events (e.g., micronucleated immature erythrocytes) have a background incidence of 0.1% (p=0.001) and 5000 cells (n=5000) are scored per animal, then the probability of detecting no spontaneously arising immature erythrocytes with micronuclei in an animal will be rare (<0.7% of animals). This determination is based upon the Poisson distribution of counts with a mean (np) of 5 where less that 0.7% of animals are expected to have a zero count. The Poisson distribution also approximates to a normal distribution when np ≥5.

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