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Standard Guide for Conduct of Micronucleus Assays in Mammalian Bone Marrow Erythrocytes[1]

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1. Scope

1.1 This guide provides recommended guidelines for performing the mammalian *in vivo* bone marrow micronucleus assay. Under appropriate test conditions, measurement of the frequency of newly formed micronucleated erythrocytes in bone marrow provides a convenient index of chromosomal damage in nucleated erythrocyte precursor cells. The rationale for the occurrence of micronuclei in conjunction with chromosomal damage has been described previously (**1**).[2] This guide describes conditions under which the frequency of micronucleated erythrocytes in mammalian bone marrow is an appropriate measure of *in vivo* chromosomal damage, and provides guidelines for the design and technical execution of assays employing this endpoint.

1.2 The following guidelines for mammalian bone marrow erythrocyte micronucleus assays have been published by organizations concerned with the evaluation of genotoxicity test data. These references should be consulted for recommendations on details not covered in depth by this guide and for requirements of specific organizations or government agencies (**2-6**).

1.3 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.4 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Summary of Guide

2.1 Animals are exposed either acutely or chronically to a test substance. At predetermined times after or during exposure, animals are sacrificed and the bone marrow is extracted, spread on slides, and stained. The frequency of micronucleated cells among the newly-formed (RNA-containing) erythrocytes is determined, and this frequency is compared among treatment groups. The newly-formed erythrocytes are identified by staining the residual RNA which remains in the newly-formed cells for about 2 days after enucleation. Cells that stain uniformly positive for RNA are referred to as polychromatic, or polychromatophilic, erythrocytes (PCEs). Cells that do not stain positively for RNA are referred to as normochromatic erythrocytes (NCEs). An increase in the frequency of micronucleated PCEs relative to the vehicle control group indicates that the test substance induced structural chromosomal damage or lagging chromosomes aneuploidy in the nucleated erythrocytic cells.

3. Significance and Use

3.1 This guide provides guidelines for the selection of animal species, dosage and sampling conditions, sampling and scoring methods, statistical design, and analysis of genotoxicity assays in which the endpoint measured is the frequency of micronucleated erythrocytes in mammalian bone marrow.

4. Animal Selection and Care

4.1 Laboratory species that are suitable for use in this assay include the mouse (*Mus musculus*), rat (*Rattus rattus*), and Chinese hamster (*Cricetulus griseus*) (**1**). Other species probably are equally suitable. If species or strains not previously used are employed, it must be established that the preparation procedure adequately visualizes RNA-containing erythrocytes and micronuclei, that potential artifacts such as aggregated RNA and mast cell granules do not interfere with the identification of micronuclei under the conditions employed, and that the micronucleus frequency is responsive to known clastogens and aneuploidy-inducing agents in that species and strain.

4.2 In choosing the species and strain of test animal, consideration should be given both to the availability of historical data on the response of that species and strain to known genotoxins and to the availability of other toxicity data on the same test material in the species and strain chosen. Choice of the same strain to be used in other genotoxicity assays of the same test material, or in long-term toxicity or

[1] This guide is under the jurisdiction of ASTM Committee F04 on Medical and Surgical Materials and Devicesand is the direct responsibility of Subcommittee F04.16 on Biocompatibility Test Methods.

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[2] The boldface numbers in parentheses refer to the list of references at the end of this guide.

carcinogenicity bioassays, has the advantage that the micronucleus frequency can be directly compared with other endpoints. The species for which the largest data base on known genotoxins is available is the mouse (**1**).

4.3 Animals should be obtained from a recognized source of laboratory animals and should be acclimated to laboratory conditions prior to use. Upon arrival, the age, sex, weight, and health of each animal should be documented. Only healthy animals should be used. Animal care and housing should conform to prevailing guidelines for the country and institution where the work is conducted. General information on guidelines for animal care and use can be obtained from the American Association for Accreditation of Laboratory Animal Care.[3] For any given experiment, all animals should be from the same source and should be approximately the same age (within one week for young adults). In the absence of special requirements for a particular age and sex, young adults of both sexes are recommended. Data from each sex should be analyzed independently.

5. Route of Administration and Choice of Vehicle

5.1 The choice of exposure route depends on the objective of the experiment. The objective of most micronucleus assays is to determine if the test substance induces types of chromosomal damage known to result in the formation of micronuclei. In this case, it is desirable to choose a route of administration and a vehicle that maximize the dose delivered to the target tissue. For this purpose, intraperitoneal and oral routes have been used most commonly, although others may also be appropriate. In other cases, the objective may be to evaluate specifically *in vivo* activity under conditions based upon known exposure routes in man. In such cases, the appropriate route is the one that provides the best experimental model of the expected exposure route in man.

5.2 The choice of a solvent or vehicle is influenced by several factors, including the chemical nature and solubility of the test substance, its toxicity to the test organism, and the route of exposure. In all cases care must be taken to ensure that the vehicle selected will not produce measurable toxicity or interfere with the normal uptake and metabolism of the test substance at the dose employed. In particular, the vehicle should not alter the spontaneous micronucleus frequency. If possible, it is desirable to use isotonic saline for parenteral administration and water or isotonic saline for oral administration. For oral administration of organic substances not readily soluble in aqueous solution, a pharmaceutical grade of corn or other vegetable oil may be used. Vegetable oil is less suitable for intraperitoneal administration because it is poorly absorbed from the peritoneal cavity. Other acceptable choices of vehicle include carboxymethylcellulose or suspension in gum arabic. Dimethylsulfoxide (DMSO) is an effective solvent for a wide range of substances and has frequently been used in experiments with mice, although there are a few reports of foreign intermediates being produced by interaction of DMSO with certain test substances (**7**) and one unconfirmed report that DMSO increases the frequency of chromosomal aberrations in the rat (**8**).

6. Dose Selection

6.1 The doses to be employed should be selected on the basis of either toxicity data obtained in the same laboratory or published toxicity data, if available. Preliminary range-finding experiment(s) should employ a minimum of two animals per dose group and should use the solvent and route of exposure to be employed in the final experiment. The highest dose level should be chosen to meet one or more of the following criteria in the experiment carried out with the full test group:

6.1.1 It should cause a marked and significant increase in the micronucleus frequency in the target cell population.

6.1.2 It should produce a statistically significant suppression of the frequency of RNA-positive erythrocytes.

6.1.3 It should cause compound-related signs of toxicity or significantly reduce survival.

6.1.4 It should be the maximum practical dose that can be administered. The maximum practical dose of a nontoxic test material is determined by the physical bulk and solubility. Testing at such a maximum dose level has been referred to as a "limit test" in OECD and EPA/TSCA testing guidelines. This dose will vary with test agent, but will generally be in the range 5 to 10 g/kg for acute oral or intraperitoneal (i.p.) administration (**3**, **5**).

6.2 The doses employed should include a minimum of two, and preferably three, doses, at least one of which does not severely reduce the frequency of RNA-positive erythrocytes (the frequency should be at least 10 to 20 % of the control value) and which does not significantly reduce the survival of the test animals. The rationale for selecting test doses has previously been discussed in the U.S. Environmental Protection Agency Gene-Tox Program report on the bone marrow polychromatic erythrocyte assay (**1**) and by Salamone and Heddle (**9**). Because the maximum cytogenetic effect is likely to be found at doses near the maximum tolerated dose (MTD), the lower doses should be spaced at relatively small increments below the highest dose (for example, no more than ½ and ¼ of the upper dose).

7. Controls

7.1 *Vehicle or Solvent Control*—A vehicle or solvent control shall be included for each sampling condition (dose, time, sex) in each experiment. Animals are treated with the solvent or vehicle in the absence of the test substance. The quantity of solvent or vehicle administered should be equivalent to the maximum given to the animals receiving the test substance. This control helps discriminate any test-substance effect from any that may have been induced by the solvent.

7.2 *Untreated Control*—The use of untreated animals is generally not necessary during routine testing. It is important, however, that each laboratory determine the frequency of micronucleated cells in animals treated with the vehicle or solvent control relative to the spontaneous frequency in untreated animals, so that any effect of the vehicle or solvent is known.

[3] Available from American Association for Accreditation of Laboratory Animal Care (AAALAC), 5283 Corporate Dr., Suite 203, Frederick, MD 21703–2879.

7.3 *Positive Control Substance*—A positive control substance, that is, a substance known to induce micronuclei in bone marrow, should be included with each experiment to confirm that all features of the protocol have been carried out correctly. The positive control agent preferably should be one that is chemically related to the test substance and preferably administered by the same route as the test article. In addition, the agent or dose should be chosen to produce a mild or weakly positive result. This provides a better evaluation of the sensitivity of the assay than does the use of a high dose of a potent clastogen which would almost always be detected regardless of whether or not the sensitivity of the assay were optimal.

8. Number of Animals/Sex

8.1 It is desirable to have data for both sexes. For routine screening, both sexes should be tested using a minimum of five animals of each sex at each test dose. If a positive result is obtained in one sex, a test agent may be classified as active without data from the other sex, but both sexes must be tested to verify a negative result.

9. Treatment and Sampling Schedule

9.1 The main requirement of the treatment/sampling schedule is to obtain at least one sample at or near the time of the maximum incidence of micronucleated cells among the RNA-positive erythrocytes in bone marrow. The time of maximum incidence varies with the test agent, dose, and treatment schedule.

9.2 *Treatment Schedule:*

9.2.1 Treatment protocols using single, double, and multiple treatments have been reported (**9**). Although each of these treatment schedules has been reported to be advantageous with specific test agents, there is insufficient evidence at present to support the exclusive use of a specific treatment schedule for all test substances. Accordingly, the choice of single, multiple, or continuous dosing protocols must be made by the investigator, based on the specific objectives of a particular study and the available knowledge of the pharmacokinetic behavior of the test substance. The use of a single- or double-dose treatment has the advantage that these protocols have been most often employed in studies reported to date, so a larger comparative data base will be available if these treatment schedules are used.

9.2.2 Although the interval between multiple treatments can affect the response obtained, little data are available to support the choice of an optimum interval. Since historical data on multiple-treatment schedules in the mouse and rat are based primarily on a 24-h dosing interval, it appears best to use this interval until definitive data supporting an alternative are available.

9.3 *Sampling Schedule:*

9.3.1 Following each treatment, there is a particular time interval during which micronucleated RNA-positive erythrocytes, if induced, would be present. Since micronuclei are formed during division of the nucleated erythropoietic cells but scored in the anucleate mature erythrocyte, micronuclei cannot appear earlier after treatment than the interval between completion of the final erythroblast mitosis and enucleation. In the mouse, this minimum time between treatment and appearance of micronuclei is about 5 h (**10**). For most chemicals, substantial increases in the micronucleus frequency have not been found earlier than 9 to 12 h after treatment. Since the life span of the RNA-positive erythrocyte within the bone marrow has been reported to be between 10 and 30 h in the mouse and rat (for review, see (**9**)), any micronucleated RNA-positive erythrocytes formed will remain in the bone marrow for at least 10 to 12 h. It is therefore not necessary to sample earlier than 19 to 24 h after the first treatment.

9.3.2 Due to differences between test agents in the time after treatment at which the peak frequency of micronuclei occurs, it is important that two or more samples be taken if only one or two treatments are given. Available data indicate that this peak frequency usually occurs between 24 and 48 h after treatment, but that in certain cases it may occur as late as 72 h after treatment (**9**). The interval between samples should be shorter than the time it would take a clastogen-affected cell population to pass through the scorable stage of erythropoiesis. This time period is approximately 24 to 36 h in mice and rats. Since a clastogen may affect more than a single erythroblast cell cycle, the period during which micronucleated PCEs are observable may be longer than 24 to 36 h (**9**). However, the micronucleated PCE frequency usually is not constant during this period, but rises to a maximum and then declines. Because it is desirable to sample as near as possible to the time of the maximum micronucleated PCE frequency, it is recommended that the time between samples not exceed approximately 24 h.

9.3.3 Based on these considerations, the following sampling schedules are recommended for experiments with mice and rats.

9.3.3.1 If one treatment is employed, a minimum of three samples should be obtained between 20 and 72 h after the treatment.

9.3.3.2 If two treatments are employed, a minimum of two samples should be obtained between 20 and 48 h after the last dose. If only two samples are taken, sampling times of approximately 20 and 48 h after the last dose would be suitable for detection of most chemicals currently known to induce micronuclei.

9.3.3.3 If three or more consecutive daily treatments are given, a single sample obtained approximately 18 to 24 h after the last dose should be sufficient.

10. Sample Preparation and Staining

10.1 *Harvesting Bone Marrow and Preparation of Smears:*

10.1.1 The principal requirements of the method of obtaining bone marrow and preparing a cell smear are the following:

10.1.1.1 A representative sample of bone marrow is obtained from each animal.

10.1.1.2 Normal cell morphology is preserved during sample preparation.

10.1.1.3 The cells are spread in a thin layer that allows individual cells to be visualized.

10.1.1.4 The cell preparation is suitable for staining in a manner that allows differentiation of the RNA-containing erythrocytes from the older erythrocytes lacking RNA, and the unequivocal identification of chromatin-containing micronuclei.