Adopted:
21st July 1997

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Mammalian Erythrocyte Micronucleus Test

INTRODUCTION

1. 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 by analysis of erythrocytes as sampled in bone marrow and/or peripheral blood cells of animals, usually rodents.

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

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

4. Definitions used are set out in the Annex.

INITIAL CONSIDERATIONS

5. The bone marrow of rodents is routinely used in this test since polychromatic erythrocytes are produced in that tissue. The measurement of micronucleated immature (polychromatic) erythrocytes in peripheral blood is equally acceptable in any species in which the inability of the spleen to remove micronucleated erythrocytes has been demonstrated, or which has shown an adequate sensitivity to detect agents that cause structural or numerical chromosome aberrations. Micronuclei can be distinguished by a number of criteria. These include identification of the presence or absence of a kinetochore or centromeric DNA in the micronuclei. The frequency of micronucleated immature (polychromatic) erythrocytes is the principal endpoint. The number of mature (normochromatic) erythrocytes in the peripheral blood that contain micronuclei among a given number of mature erythrocytes can also be used as the endpoint of the assay when animals are treated continuously for 4 weeks or more.

6. This mammalian *in vivo* micronucleus test is especially relevant to assessing mutagenic hazard in that it allows consideration of factors of *in vivo* metabolism, pharmacokinetics and DNA-repair processes although these may vary among species, among tissues and among genetic endpoints. An *in vivo* assay is also useful for further investigation of a mutagenic effect detected by an *in vitro* system.

7. If there is evidence that the test substance, or a reactive metabolite, will not reach the target tissue, it is not appropriate to use this test.

PRINCIPLE OF THE TEST METHOD

8. Animals are exposed to the test substance by an appropriate route. If bone marrow is used, the animals are sacrificed at appropriate times after treatment, the bone marrow extracted, and preparations made and stained (1)(2)(3)(4)(5)(6)(7). When peripheral blood is used, the blood is collected at appropriate times after treatment and smear preparations are made and stained (4)(8)(9)(10). For studies with peripheral blood, as little time as possible should elapse between the last exposure and cell harvest. Preparations are analyzed for the presence of micronuclei.

DESCRIPTION OF THE METHOD

Preparations

Selection of animal species

9. Mice or rats are recommended if bone marrow is used, although any appropriate mammalian species may be used. When peripheral blood is used, mice are recommended. However, any appropriate mammalian species may be used provided it is a species in which the spleen does not remove micronucleated erythrocytes or a species which has shown an adequate sensitivity to detect agents that cause structural or numerical chromosome aberrations. Commonly used laboratory strains of young healthy animals should be employed. 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.

Housing and feeding conditions

10. The temperature in the experimental animal room should be 22°C (±3°C). Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning, the aim should be 50-60%. 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. Animals may be housed individually, or caged in small groups of the same sex.

Preparation of the animals

11. Healthy young adult animals are randomly assigned to the control and treatment groups. The animals are identified uniquely. The animals are 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.

Preparation of doses

12. Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted, if appropriate, prior to dosing of the animals. Liquid test substances may be dosed directly or diluted prior to dosing. Fresh preparations of the test substance should be employed unless stability data demonstrate the acceptability of storage.

Test conditions

Solvent/vehicle

13. The solvent/vehicle should not produce toxic effects at the dose levels used, and should not be suspected 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

14. Concurrent positive and negative (solvent/vehicle) controls should be included for each sex in each test. Except for treatment with the test substance, animals in the control groups should be handled in an identical manner to animals of the treatment groups.

15. Positive controls should produce micronuclei *in vivo* at exposure levels expected to give a detectable increase over background. Positive control doses should be chosen so that the effects are clear but do not immediately reveal the identity of the coded slides to the reader. It is acceptable that the positive control be administered by a route different from the test substance and sampled at only a single time. In addition, the use of chemical class-related positive control chemicals may be considered, when available. Examples of positive control substances include:

Chemical and CAS No.
Ethyl methanesulphonate [CAS no. 62-50-0]
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]

16. Negative controls, treated with solvent or vehicle alone, and otherwise treated in the same way as the treatment groups should be included for every sampling time**,** unless acceptable inter-animal variability and frequencies of cells with micronuclei are demonstrated by historical control data. If single sampling is applied for negative controls, the most appropriate time is the first sampling time. In addition, untreated controls should also be used unless there are historical or published control data demonstrating that no deleterious or mutagenic effects are induced by the chosen solvent/vehicle.

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

PROCEDURE

Number and sex of animals

18. Each treated and control group must include at least 5 analysable animals per sex (11). If at the time of the study there are data available from studies in the same species and using the same route of exposure that demonstrate that there are no substantial differences between sexes in toxicity, then testing in a single sex will be sufficient. 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

19. No standard treatment schedule (i.e. 1, 2, or more treatments at 24 h intervals) can be recommended. The samples from extended dose regimens are acceptable as long as a positive effect has been demonstrated for this study or, for a negative study, as long as toxicity has been demonstrated or the limit dose has been used, and dosing continued until the time of sampling. Test substances may also 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.

20. The test may be performed in two 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. 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 recognized at one sampling time, additional sampling is not required.

(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 36 and 48 hours following the final treatment for the peripheral blood (12).

21. Other sampling times may be used in addition, when relevant.

Dose levels

22. If a range finding study is performed because there are no suitable data available, it should be performed in the same laboratory, using the same species, strain, sex, and treatment regimen to be used in the main study (13). If there is toxicity, three dose levels are used for the first sampling time. These dose levels should cover a range from the maximum to little or no toxicity. At the later sampling time only the highest dose needs to be used. The highest dose is defined as the dose producing signs of toxicity such that higher dose levels, based on the same dosing regimen, would be expected to produce lethality. Substances with specific biological activities at low non-toxic doses (such as hormones and mitogens) may be exceptions to the dose-setting criteria and should be evaluated on a case-by-case basis. The highest dose may also be defined as a dose that produces some indication of toxicity of the bone marrow (e.g. a reduction in the proportion of immature erythrocytes among total erythrocytes in the bone marrow or peripheral blood).

Limit test

23. If a test at one dose level of at least 2000 mg/kg body weight using a single treatment, or as two treatments on the same day**,** produces no observable toxic effects**,** and if genotoxicity would not be expected based upon data from structurally related substances, then a full study using three dose levels may not be considered necessary. For studies of a longer duration, the limit dose is 2000 mg/kg/body weight/day for treatment up to 14 days, and 1000 mg/kg/body weight/day for treatment longer than 14 days. Expected human exposure may indicate the need for a higher dose level to be used in the limit test.

Administration of doses

24. The test substance is usually administered by gavage using a stomach tube or a suitable intubation cannula, or by intraperitoneal injection. Other routes of exposure may be acceptable where they can be justified. 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 higher than these must be justified. Except for irritating or corrosive substances which will normally reveal exacerbated effects with higher concentrations, variability in test volume should be minimised by adjusting the concentration to ensure a constant volume at all dose levels.

Bone marrow / blood preparation

25. Bone marrow cells are usually obtained from the femurs or tibias immediately following sacrifice. Commonly, cells are removed from femurs or tibias, prepared and stained using established methods. Peripheral blood is obtained from the tail vein or other appropriate blood vessel. Blood cells are immediately stained supravitally (8)(9)(10) or smear preparations are made and then stained. The use of a DNA specific stain [e.g. acridine orange (14) or Hoechst 33258 plus pyronin-Y (15)] 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). Additional systems [e.g. cellulose columns to remove nucleated cells (16)] can also be used provided that these systems have been shown to adequately work for micronucleus preparation in the laboratory.

Analysis

26. 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 (17). All slides, including those of positive and negative controls, should be independently coded before microscopic analysis. At least 2000 immature erythrocytes per animal are scored for the incidence of micronucleated immature erythrocytes. Additional information may be obtained by scoring mature erythrocytes for micronuclei. When analysing slides, the proportion of immature erythrocytes among total erythrocytes should not be less than 20% of the control value. When animals are treated continuously for 4 weeks or more, at least 2000 mature erythrocytes per animal can also be scored for the incidence of micronuclei. Systems for automated analysis (image analysis and cell suspensions flow cytometry) are acceptable alternatives to manual evaluation if appropriately justified and validated .

DATA AND REPORTING

Treatment of results

27. Individual animal data should be presented in tabular form. The experimental unit is the animal. 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 animals are treated continuously for 4 weeks or more, the data on mature erythrocytes should also be given if it is collected. The proportion of immature among total erythrocytes and, if considered applicable, the percentage of micronucleated erythrocytes is given for each animal. If there is no evidence for a difference in response between the sexes, the data from both sexes may be combined for statistical analysis.

Evaluation and interpretation of results

28. There are several criteria for determining a positive result, such as a dose-related increase in the number of micronucleated cells or a clear increase in the number of micronucleated cells in a single dose group at a single sampling time. Biological relevance of the results should be considered first. Statistical methods may be used as an aid in evaluating the test results (18)(19). Statistical significance should not be the only determining factor for a positive response. Equivocal results should be clarified by further testing preferably using a modification of experimental conditions.

29. A test substance for which the results do not meet the above criteria is considered non-mutagenic in this test.

30. Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance. Results, may remain equivocal or questionable regardless of the number of times the experiment is repeated. 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. Negative results indicate that, under the test conditions, the test substance does not produce micronuclei in the immature erythrocytes of the test species.

31. The likelihood that the test substance or its metabolites reach the general circulation or specifically the target tissue (e.g. systemic toxicity) should be discussed.

Test report

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

Test substance:

- identification data and CAS no., if known;
- physical nature and purity;
- physiochemical 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.

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 of administration;
- methods for verifying that the test substance reached the general circulation or target tissue, if applicable;
- conversion from diet/drinking water test substance concentration (ppm) to the actual dose (mg/kg body weight/day), if applicable;
- details of food and water quality;
- detailed description of treatment and sampling schedules;
- methods of slide preparation;
- 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.

Results:

- 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;
- concurrent positive control data.

Discussion of the results.

Conclusion.

LITERATURE

(1) Heddle, J.A. (1973). A Rapid *In Vivo* Test for Chromosomal Damage, Mutation Res., 18, 187-190.

(2) Schmid, W. (1975). The Micronucleus Test, Mutation Res., 31, 9-15.

(3) Heddle, J.A., Salamone, M.F., Hite, M., Kirkhart, B., Mavournin, K., MacGregor, J.G. and Newell, G.W. (1983). The Induction of Micronuclei as a Measure of Genotoxicity. Mutation Res. 123, 61-118.

(4) Mavournin, K.H., Blakey, D.H., Cimino, M.C., Salamone, M.F. and Heddle, J.A. (1990). The *In Vivo* Micronucleus Assay in Mammalian Bone Marrow and Peripheral Blood. A report of the U.S. Environmental Protection Agency Gene-Tox Program, Mutation Res., 239, 29-80.

(5) MacGregor, J.T., Schlegel, R. Choy, W.N., and Wehr, C.M. (1983). Micronuclei in Circulating Erythrocytes: A Rapid Screen for Chromosomal Damage During Routine Toxicity Testing in Mice. in: "Developments in Science and Practice of Toxicology", Ed. A.W. Hayes, R.C. Schnell and T.S. Miya, Elsevier, Amsterdam, pp. 555-558.

(6) MacGregor, J.T., Heddle, J.A., Hite, M., Margolin, G.H., Ramel C., Salamone, M.F., Tice, R.R. and Wild, D. (1987). Guidelines for the Conduct of Micronucleus Assays in Mammalian Bone Marrow Erythrocytes. Mutation Res., 189, 103-112.

(7) MacGregor, J.T., Wehr, C.M., Henika, P.R., and Shelby, M.E. (1990). The *in vivo* Erythrocyte Micronucleus Test: Measurement at Steady State Increases Assay Efficiency and Permits Integration with Toxicity Studies. Fund. Appl. Toxicol., 14, 513-522.

(8) Hayashi, M., Morita, T., Kodama, Y., Sofuni, T., and Ishidate, M. Jr. (1990). The Micronucleus Assay with Mouse Peripheral Blood Reticulocytes Using Acridine Orange-Coated Slides. Mutation Research, 245, 245-249.

(9) The Collaborative Study Group for the Micronucleus Test (1992). Micronucleus Test with Mouse Peripheral Blood Erythrocytes by Acridine Orange Supravital Staining: The Summary Report of the 5th Collaborative Study by CSGMT/JEMS. MMS. Mutation Res., 278, 83-98.

(10) The Collaborative Study Group for the Micronucleus Test (CSGMT/JEMMS.MMS, The Mammalian Mutagenesis Study Group of the Environmental Mutagen Society of Japan) (1995). Protocol recommended for the short-term mouse peripheral blood micronucleus test. Mutagenesis, 10, 153-159.

(11) Hayashi, M., Tice, R.R., MacGregor, J.T., Anderson, D., Blakey, D.H., Kirsch-Volders, M., Oleson, Jr. F.B.., Pacchierotti, F., Romagna, F., Shimada, H., Sutou, S. and Vannier, B. (1994). *In Vivo* Rodent Erythrocyte Micronucleus Assay. Mutation Res., 312, 293-304.

(12) Higashikuni, N. and Sutou, S. (1995). An optimal, generalized sampling time of 30 +/- 6 h after double dosing in the mouse peripheral blood micronucleus test. Mutagenesis, 10, 313-319.

(13) Fielder, R. J., Allen, J. A., Boobis, A. R., Botham, P. A., Doe, J., Esdaile, D. J., Gatehouse, D. G., Hodson-Walker, G., Morton, D. B., Kirkland, D. J., and Richold, M. (1992). Report of British Toxicology Society/UK Environmental Mutagen Society Working Group: Dose Setting in *In Vivo* Mutagenicity Assays. Mutagenesis, 7, 313-319.

(14) Hayashi, M., Sofuni, T., and Ishidate, M. Jr. (1983). An Application of Acridine Orange Fluorescent Staining to the Micronucleus Test. Mutation Res., 120, 241-247.

(15) MacGregor, J. T., Wehr, C. M., and Langlois, R. G. (1983). A Simple Fluorescent Staining Procedure for Micronuclei and RNA in Erythrocytes Using Hoechst 33258 and Pyronin Y. Mutation Res., 120, 269-275.

(16) Romagna, F. and Staniforth, C.D. (1989). The automated bone marrow micronucleus test. Mutation Res., 213, 91-104.

(17) Gollapudi, B. and McFadden, L.G. (1995). Sample size for the estimation of polychromatic to normochromatic eruthrocyte ratio in the bone marrow micronucleus test. Mutation Res., 347, 97-99.

(18) Richold, M., Ashby, J., Bootman, J., Chandley, A., Gatehouse, D.G. and Henderson, L. (1990). *In Vivo* Cytogenetics Assays, In: D.J.Kirkland (Ed.) Basic Mutagenicity Tests, UKEMS Recommended Procedures. UKEMS Subcommittee on Guidelines for Mutagenicity Testing. Report. Part I revised. Cambridge University Press, Cambridge, New York, Port Chester, Melbourne, Sydney, pp. 115-141.

(19) Lovell, D.P., Anderson, D., Albanese, R., Amphlett, G.E., Clare, G., Ferguson, R., Richold, M., Papworth, D.G.and Savage, J.R.K. (1989). Statistical Analysis of *In Vivo* Cytogenetic Assays In: D.J. Kirkland (Ed.) Statistical Evaluation of Mutagenicity Test Data. UKEMS Sub-Committee on Guidelines for Mutagenicity Testing, Report, Part III. Cambridge University Press, Cambridge, New York, Port Chester, Melbourne, Sydney, pp. 184-232.

ANNEX

DEFINITIONS

Centromere (Kinetochore): 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.

Normochromatic erythrocyte: mature erythrocyte that lacks ribosomes and can be distinguished from immature, polychromatic erythrocytes by stains selective for ribosomes.

Polychromatic erythrocyte: immature erythrocyte, in an intermediate stage of development, that still contains ribosomes and therefore can be distinguished from mature, normochromatic erythrocytes by stains selective for ribosomes.