

Short-Term Screening Method for the Prediction of Carcinogenicity of Chemical Substances: Current Status and Problems of an *in vivo* Rodent Micronucleus Assay

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(Received September 8, 2000)

Various short-term screening methods have been developed to detect mutagenic/carcinogenic substances. They have played important roles not only in screening suspected chemicals but in studying the mechanisms of mutagenesis/carcinogenesis, and have provided useful information for assessing the genetic effects of chemicals on humans. The micronucleus assay is an *in vivo* mammalian methods, which has been widely used for screening the genotoxic potency of chemical substances. By this assay, the genotoxicity of chemicals, including clastogens and spindle poisons, have been evaluated using immature bone marrow erythrocytes in mice. The use of immature erythrocytes in circulating peripheral blood instead of in bone marrow of mice has been developed in recent years and it has been determined that it gives results as relevant as those of bone marrow cells in mice. Using the peripheral blood of mice has made the micronucleus assay method more practical and useful. Recently, rats have been identified as an acceptable species for this assay, and assays using the peripheral blood of rats are often carried out concomitantly with general toxicity or carcinogenic studies. However, it must be noted that the micronucleus assays have some limitations. The activity of substances which are metabolized rapidly in mice or rats, for example, may hardly be detected by the micronucleus assay; metabolic features of the substances may affect the assay results greatly. In this review, the current status of the micronucleus assay is discussed as a short-term screening method in the context of its usefulness and its limitations.

Key words — *in vivo* micronucleus assay, carcinogenicity screening, bone marrow, peripheral blood, mouse, rat

INTRODUCTION

Mutations occur spontaneously or under the influence of external factors, such as chemical substances or ionizing radiation. Monitoring and evaluating the activities of the mutagenic substances in the environment are important, as they often induce genetic toxicities. Various *in vitro* and *in vivo* short-term mutagenicity assays have been developed to detect genotoxic active substances, some of which are carcinogenic to humans. *In vitro* bacterial short-term assay methods using several strains of *Salmonella typhimurium* were developed by Ames *et al.*,^{1–3)}

and are called Ames assay. In this assay, substances with mutagenic activity are conveniently tested in a short period of time, and there is a fairly high correlation between the mutagenicity and carcinogenicity of the chemical substances tested. There are a number of short-term mutagenicity testing methods⁴⁾ *in vivo* as well as *in vitro*, such as gene mutation assay, chromosome aberration assay and DNA damage assay. These mutagenicity tests have been used for the detection of mutagens, and the results of the tests are sometimes used to understand the fundamental mechanisms of chemical carcinogenesis.⁵⁾ The micronucleus assay^{6–8)} using immature bone marrow erythrocytes of mice has been widely used as a simple and sensitive short-term screening method *in vivo* for determining the mutagenicity of chemical substances. As this assay uses “whole animals”, it has the merits of including such factors as

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absorption, distribution, and metabolism of the chemical substances in the evaluation. The micronucleus assay using immature erythrocytes in circulating peripheral blood has begun to be used recently.

In this review, the current situation and problems of the micronucleus assay are described and discussed in view of their usefulness and limitations as a short-term animal assay to detect the genotoxicity of chemicals.

1. Mouse Bone Marrow Micronucleus Assay as an *in vivo* Mutagenicity Test

The mouse bone marrow micronucleus assay (see Fig. 1 for the procedure)⁶⁻⁸ is based on the detection of the small nucleus (micronucleus) formed from chromosomal damage by chemical substances. The formed micronuclei remain in the cytoplasm. These micronuclei are formed by clastogenic substances and spindle poisons (see Fig. 2). When the forming function of the spindle body is obstructed, a micronucleus occurs with one to several chromosomes. Therefore, whole chromosomes containing micronuclei are observed as large size fragments rather than lagging chromosome fragments.⁹ Recently, a molecular cytogenetic method, *i.e.*, "fluorescent *in site* hybridization (FISH)",^{10,11} with centromere DNA-probes were developed. By this method, the presence of centromeres in micronuclei can be clearly detected, and the ability to detect differences between the micronucleus induced by clastogens or by spindle poisons became possible.¹²

Extensive studies of clastogens by micronucleus assay have led to the following conclusions: 1) any mouse and rat strain is acceptable; 2) one sex, either male or female can be used; 3) treatment by either intraperitoneal injection or oral administration is acceptable; 4) examination 24–48 hr after a single administration of at least one dose will be acceptable to evaluate the mutagenicity of chemicals.¹³

Recently, the acridine orange (AO) fluorescent staining method has been introduced instead of the Giemsa (G) staining method to improve the identification of immature erythrocytes.¹⁴ The AO staining method gives more reliable results than the usual G staining method in the micronucleus assay. By this AO fluorescent staining method, both immature erythrocytes and a micronucleus can be easily distinguished from the mature erythrocytes. It is generally recognized that the AO fluorescent staining method is more useful than the G method for obtaining reliable data in the micronucleus assay.

Introduction of the AO staining method demon-

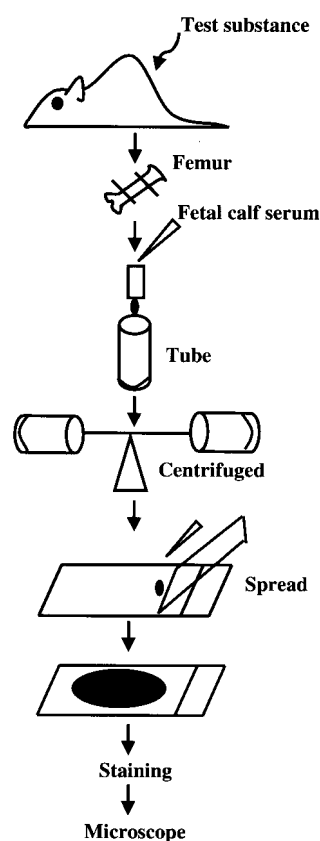


Fig. 1. Bone Marrow Micronucleus Test in Rodent

The animals were killed 24–48 hr after the dosing of test substances. The bone marrow cells of the femur were collected and flushed out with fetal calf serum and centrifuged. The pellets were spread on a slide glass and fixed with methanol, then stained.

strated that it could be applied to rats as well as mice. Micronucleus assay using rats had a disadvantage: when specimens from rats were spread large numbers of granules from mast cell were developed over the slide, and some of them were accidentally superimposed on the erythrocytes. These micronuclei and granules, stained similarly blue by G staining, were occasionally misjudged to all be micronuclei. The AO fluorescent staining method is applicable to rats in the bone marrow micronucleus assay; with this method it is easy to distinguish a micronucleus from mast-cell granules.¹⁵ Rats are the most widely used animals for general toxicologic, carcinogenic, pharmacokinetic and toxico-kinetic studies. Many data are available and would be useful for the overall assessment of chemical substances. Currently, the rat micronucleus assay is one of the most important methods to evaluate the genetic toxicity of chemicals *in vivo*.

The current status of the micronucleus assay will be discussed further for its usefulness and limitations.

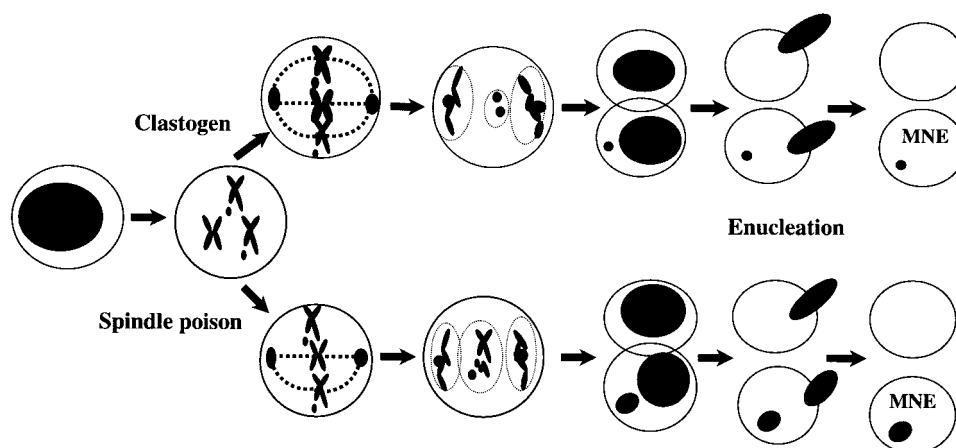


Fig. 2. Formation of Micronucleated Erythrocytes by Mutagens

Chromosomal structure aberration occurs in erythroblasts as a result of exposure to harmful chemical substances. In anaphase, the chromosome fragments lag behind when the centric elements move towards the spindle poles. After telophase, a small nucleus (micronucleus) is formed from the chromosomal fragments. In enucleation, the micronucleus remains in the cytoplasm, making it more visible in the erythrocytes. Similar events occur if the functioning of the spindle apparatus is impaired; in general, a micronucleus thus formed is considerably larger than a topical micronucleus. MNE: Micronucleated erythrocytes.

2. The Induction of a Micronucleus by Carcinogenic Substances

The bone marrow micronucleus assay using mice has proven to be a useful method for predicting the carcinogenicity of chemical substances.

Polycyclic aromatic hydrocarbons (PAH) are chemical compounds which require metabolic activation by microsomal monooxygenase enzymes in order to become mutagenic and/or carcinogenic. Inducible levels of aryl hydrocarbon hydroxylase (AHH), which is a microsomal monooxygenase of the CYP1 family, is different among mouse strains. It has been known that there is a good positive correlation between the inducibility of AHH, and the inducibility of tumors by certain carcinogens. AHH-inducible mouse strains were more sensitive than the AHH-noninducible mouse strain in terms of tumor induction by PAH.¹⁶⁻¹⁸⁾ These findings suggest that the induction of tumors by PAH will be clearly correlated to their metabolic activation by AHH. We have compared AHH-inducible mice (BALB/c and C57BL/6) with AHH-noninducible mice (DBA/2) in terms of the results in the mouse bone marrow micronucleus assay, 2-stage skin carcinogenesis test¹⁹⁾ and single cell gel electrophoresis (SCGE) assay^{20,21)} using 7,12-dimethylbenz[*a*]anthracene (DMBA) as a reference carcinogen. SCGE assay is a useful method for detecting DNA damage by chemical substances in single cell levels. Each mouse strain was used, with 3 mice/group in the micronucleus assay, 5 mice/group in the SCGE assay and 30 mice/group in the skin carcinogenesis test, re-

spectively.

The test results are shown in Fig. 3. The AHH-inducible mice (BALB/c and C57BL/6) were more sensitive than AHH-noninducible mice (DBA/2) for DMBA in the 3 test methods. The incidences of the development of micronuclei, DNA-damaged cells and skin tumors were markedly increased in the AHH-inducible mice (BALB/c and C57BL/6), as compared with the AHH-noninducible mice (DBA/2).^{22,23)} These results suggest that carcinogenicity induced by DMBA in mice correlates well with the induction of a micronucleus.

The mouse bone marrow micronucleus assay, therefore, would be a simple and sensitive short-term method for the predicting the carcinogenicity of chemical substances.

3. Correlation Between the Micronucleus Assay and Carcinogenicity Test Results

The International Agency for Research on Cancer (IARC) issues monographs containing lists of substances that cause cancer in humans.²⁴⁾ In this monograph, the cancer causing chemicals to humans are registered in 4 categories. Group 1 (the substance is carcinogenic to humans), 2A (the substance is probably carcinogenic to humans), 2B (the substance is possibly carcinogenic to humans), 3 (the substance is unclassifiable as a carcinogen to humans) and 4 (the substance is probably not carcinogenic to humans). To assess the correlation between the micronucleus induction potency and carcinogenic activity, the micronucleus assay was performed as a col-

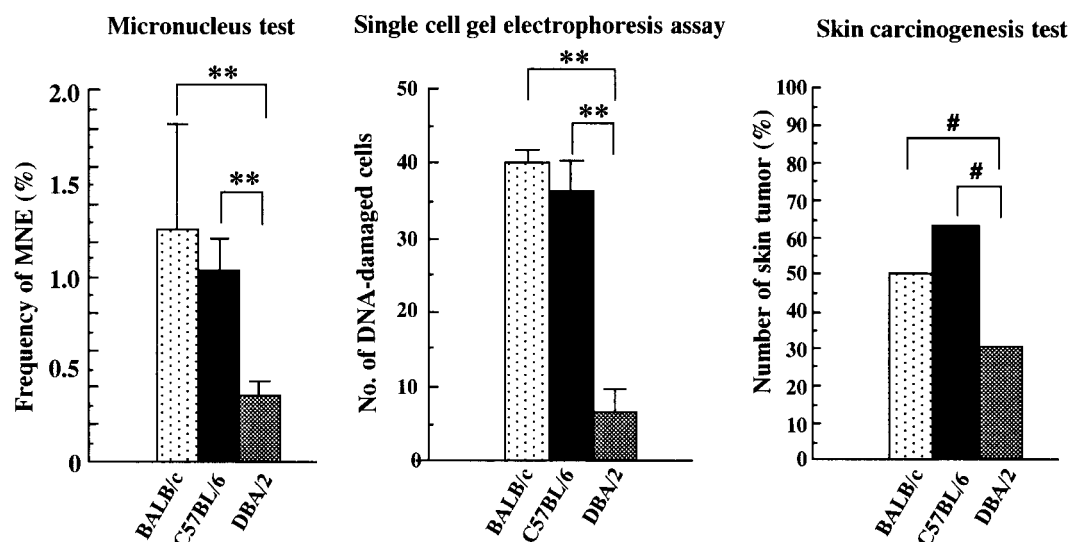


Fig. 3. Comparison of the Micronucleus Test, Single Cell Gel Electrophoresis Assay and Two-Stage Skin Carcinogenesis Test of BALB/c, C57BL/6 and DBA/2 Mice Treated with 7,12-Dimethylbenz[*a*]anthracene^{22,23)}

Micronucleus test: Mice were treated with DMBA at the dose of 100 mg/kg (*i.p.*). The bone marrow cells were collected and stained 48 hr after the DMBA treatment. Micronucleated erythrocytes (MNE) were examined in 1000 erythrocytes per mouse of each mouse strain. Single cell gel electrophoresis assay: Mice were treated with DMBA at the dose of 100 mg/kg (*i.p.*). Peripheral blood was collected from the tail vessel 12 hr after the treatment. Damaged cells were examined in 200 cells per mouse of each mouse strain. Skin carcinogenesis test: Mice were treated with DMBA at the dose of 100 μ g/mouse on the back skin. After 2 weeks, they were treated with croton oil at the dose of 15 μ g/mouse in acetone 3 times/week for 104 weeks. **: $p < 0.01$ by Student's *t*-test, #: $p < 0.05$ by χ^2 -test.

laborative study by The Collaborative Study Group for the Micronucleus Test (CSGMT, The Environmental Mutagen Society of Japan).²⁵⁾

The experimental results of the micronucleus assay were evaluated by comparing our present data with published data on the IARC carcinogens. The positive rates for groups 1, 2A and 2B were 68.6, 54.5 and 45.6%, respectively. After incorporating information on the structure-activity relationship, the positive rates of the micronucleus assay become 90.5, 65.2 and 60.0% for IARC groups 1, 2A and 2B, respectively. It must be noted that the positive rates tended to be higher in carcinogens with a higher risk for human carcinogenicity.²⁵⁾ Based upon these results, it is suggested that the use of the micronucleus assay is useful as an *in vivo* short-term screening method to predict the human carcinogenicity of chemical substances.

4. The Micronucleus Assay with Peripheral Blood

The micronucleus assay was based on examination of the micronucleus in immature bone marrow erythrocytes in rodents. The immature bone marrow erythrocytes enter circulation in the peripheral blood. A micronucleus assay with peripheral blood will thus be possible if examination of the micronucleus in immature peripheral erythrocytes is possible. By using peripheral blood, the safety evaluation of

chemical substances may be expanded from mice to rats or even to humans. The following were explored relating to a peripheral blood micronucleus assay.

4.1 The Development and Improvement of a Peripheral Blood Micronucleus Assay

—The peripheral blood micronucleus assay²⁶⁾ was first developed in mice by MacGregor *et al.* in 1980. However, the amount of immature erythrocytes in peripheral blood is so small, that micronucleus evaluation is very difficult. Moreover, because of the difficulty in distinguishing mature erythrocytes by G staining, this method has not been widely used. If the peripheral blood could be used for micronucleus assay, it may be possible to obtain time-dependent data in a dose-response manner. As in the peripheral blood assay, the same animal can be used for several samplings and it may be possible to limit the number of animals used and the amount of each substance to be tested. As a result, more useful information about micronucleus induction could be obtained compared to the bone marrow micronucleus assay. Recently, immature erythrocytes in the peripheral blood became easy to distinguish by the introduction of the AO fluorescence method developed by Hayashi *et al.* in 1990.^{14,27)} The peripheral micronucleus assay can be carried out as shown in Fig. 4. 1) AO solution is spread homogeneously on the slide

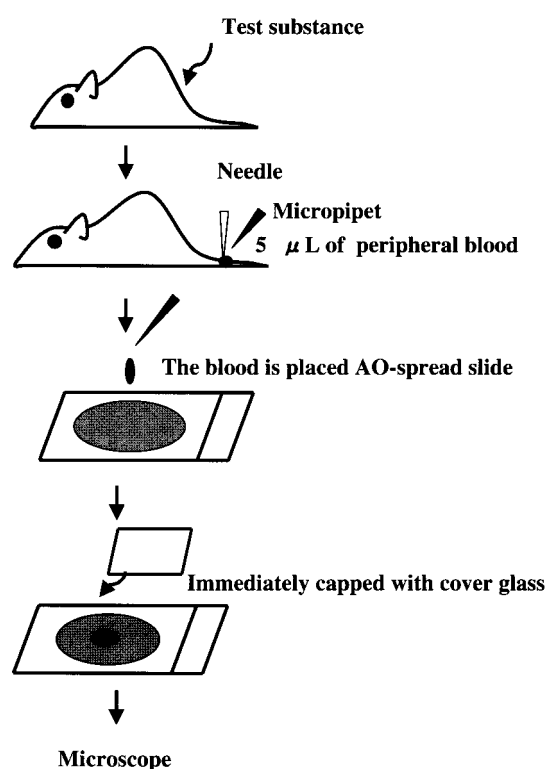


Fig. 4. Peripheral Blood Micronucleus Test with Rodents

Peripheral blood is obtained by piercing a tail blood vessel. Five μ l of the blood is placed in the center of slide glass spread with acridine orange and covered immediately with a cover glass.

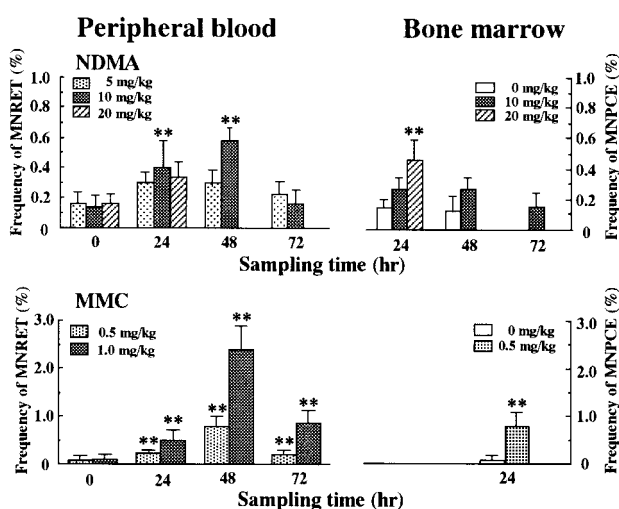


Fig. 5. Results of the Bone Marrow and Peripheral Blood Micronucleus Test

The frequency (%) of micronucleated reticulocytes (MNRET) in the peripheral blood and micronucleated polychromatic erythrocytes (MNPCE) was examined in the bone marrow of CD-1 (ICR) mice which had been treated with *N*-Nitrosodimethylamine (NDMA) or Mitomycin C (MMC). MNRET and MNPCE were examined in 1000 reticulocytes and 1000 polychromatic erythrocytes from each dose group, respectively. ** $p < 0.01$: Significant difference from the control (the pre-treatment values, 0 hr) or vehicle control.

glass (AO-coated slide), 2) 5 μ l of peripheral blood is collected by piercing a tail vessel, 3) the blood is placed without any anticoagulant on the center of an AO-coated slide and covered immediately with cover-glass.²⁷⁾ The peak frequency of micronuclei in peripheral blood immature erythrocytes is usually delayed by about 24 hr compared with that of bone marrow cells (results are shown in Fig. 5).^{28–31)} It has been shown that bone marrow cells can be replaced by peripheral cells without any problem in sensitivity.

This method using animal blood can be applied to humans, fish, shell-fish and also to insects.^{32–35)}

4.2 Application of the Micronucleus Assay Using Peripheral Blood Cells

—Rats and mice are the animals most commonly used for either long-term toxicological study or the carcinogenic study of chemical substances. If the data of ongoing long-term toxicological study would be available for micronucleus assay, it may be useful to compare all research data on toxico-kinetic, organo-toxic and carcinogenic studies of chemical substances. Such data would be important for overall toxicological assessment. The peripheral blood micronucleus assay with rats or mice has been examined and compared with the data of long-term toxicological studies.^{36–38)}

Recently, a new gene mutation assay *in vivo* was developed using *transgenic animals*, such as, MutaTMMouse³⁹⁾ or Big BlueTM.⁴⁰⁾ In that study, the animals have shuttle vectors containing the *lac Z* or *lac I* gene of *Escherichia coli*. The offspring possess the inserted DNA in all somatic and germ cells. Therefore, the *transgenic mice* contain a bacterial gene as a mutation target. A carcinogenicity test using the *transgenic mice* will take into account all the factors that may affect the induction of gene mutations, DNA-repair, chromosome aberration or carcinogenicity. By using the *transgenic mice*, all tests become possible in the same animal. It is clear that the development of *transgenic mice* marks a great advance in genotoxicity and carcinogenicity research.

5. Problems Hereafter in a Mutagenicity Test

Though the micronuclei assay is very useful as a short-term screening method, it is difficult to evaluate the safety of chemical substances based on only one short-term test system. It is recommended in the guidelines for genotoxicity studies of chemical substances that the *in vitro* bacterial reverse mutation

assay (Ames test), *in vitro* mammalian cells chromosome abnormal test and *in vivo* rodent micronucleus assay should be used, as in the battery.

The *in vivo* micronucleus assay in bone marrow cells is used to detect chromosome aberration, thus the target cells must be sufficiently exposed to the chemical substance tested. Formaldehyde (FA) is known to induce squamous cell carcinoma of the rat nasal cavity in an inhalation carcinogenesis test. FA showed genotoxicity some *in vitro* genotoxicity in a short-term assay, but not in the bone marrow micronucleus assay.³¹⁾ FA administered orally, intraperitoneally or intravenously may be inactivated before it reaches the target bone marrow cells. Therefore, the bone marrow micronucleus assay may not be suitable for clastogenic compounds, which are reactive to other cellular molecules and have difficulty reaching bone marrow cells. A micronucleus assay using other organs or tissue, such as the liver,⁴¹⁾ skin⁴²⁾ and colon epithelium,⁴³⁾ have recently been employed in addition to the bone marrow micronucleus assay. The labeling of all chromosomes by spectral karyotyping⁴⁴⁾ in the FISH method, mentioned in section 1, yields information about specific chromosomal aberrations in micronuclei, and will be possible using some DNA-probes.

Examination of micronuclei by the human eye is time-consuming. Image analysis scoring⁴⁵⁾ or flow cytometry⁴⁶⁻⁴⁸⁾ devices are improvements as automatic micronucleus scoring systems. These methods will certainly improve the speed and sensitivity of the micronucleus assay.

A micronucleus assay using the cells of bone marrow or peripheral blood (or of other tissues or organs) of small animals is convenient and is one of the most promising assay systems, especially when used with FISH methods. Scoring devices systems which are currently being developed will make this assay more useful, without doubt, for predicting the carcinogenicity of chemical substances.

Acknowledgment We thank Dr. M. Hayashi, Division of Genetics and Mutagenesis, National Institute of Health Sciences, Japan, for specific data analysis, suggestions and discussion of the micronucleus assay.

REFERENCES

- 1) Ames, B. N., Durston, W. E., Yamasaki, E. and Lee, F. D. (1973) Carcinogens are mutagens: A simple test system combining liver homogenates for activation and bacteria for detection. *Proc. Nat. Acad. Sci. (U.S.A.)*, **70**, 2281–2285.
- 2) Ames, B. N., McCann, J. and Yamasaki, E. (1975) Methods for detecting carcinogens and mutagens with the *salmonella*/mammalian-microsome mutagenicity test. *Mutation Res.*, **31**, 347–364.
- 3) McCann, J. and Ames, B. N. (1976) Detection of carcinogens as mutagens in the *Salmonella*/microsome test: Assay of 300 chemicals: Discussion. *Proc. Nat. Acad. Sci. (U.S.A.)*, **73**, 950–954.
- 4) Ministry of Health and Welfare, Japan (1991) Guideline for Toxicity Studies of Drugs “Notification No. 24 of the Pharmaceutical Affairs Bureau, Ministry of Health and Welfare, Japan, September 11, 1989”, Yakuji Nippo, Ltd., Tokyo, pp. 95–111.
- 5) Shimoi, K. and Tomita, I. (1991) Mutagens and antimutagens in the living environment. *Eisei Kagaku (J. Health Sci.)*, **37**, 149–178.
- 6) Schmid, W. (1973) Chemical mutagen testing on *in vivo* somatic mammalian cells. *Agents and Actions*, **3**, 77–85.
- 7) Heddle, J. A. (1973) A rapid *in vivo* test for chromosomal damage. *Mutation Res.*, **18**, 187–190.
- 8) Schmid, W. (1975) The micronucleus test. *Mutation Res.*, **31**, 9–15.
- 9) Yamamoto, K. I. and Kikuchi, Y. (1980) A comparison of diameters of micronuclei induced by clastogens and by spindle poisons. *Mutation Res.*, **71**, 127–131.
- 10) Becker, P., Scherthan, H. and Zankl, H. (1990) Use of a centromere-specific DNA probe (p82H) in nonisotopic *in situ* hybridization for classification of micronuclei. *Genes Chromosomes Cancer*, **2**, 59–62.
- 11) Miller, B. M., Zitzelsberger, H. F., Weier, H.-Ul. G. and Adler, I.-D. (1991) Classification of micronuclei in murine erythrocytes: Immunofluorescent staining using CREST antibodies compared to *in situ* hybridization with biotinylated gamma satellite DNA. *Mutagenesis*, **6**, 297–302.
- 12) Komae, N., Hibino, Y. and Sugano, N. (1999) Analysis of micronuclei induced under hyperthermic conditions in human lymphocyte culture by fluorescence *in situ* hybridization (FISH) and spectral karyotyping (SKY) methods. *Yakugaku Zasshi*, **119**, 763–772.
- 13) Sutou, S. (1996) Achievements by CSGMT/JEMS·MMS: The collaborative study group for the micronucleus test in the mammalian mutagenesis study group of the environmental mutagen society of Japan. *Mutation Res.*, **340**, 151–174.
- 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) Wakata, A., Miyamae, Y., Sato, S., Suzuki, T., Morita, T., Asano, N., Awogi, T., Kondo, K. and Hayashi, M. (1998) Evaluation of the rat micronucleus test with bone marrow and peripheral blood: Summary of the 9th collaborative study by CSGMT/JEMS.MMS. *Environ. Mol. Mutagen.*, **32**, 84–100.
 - 16) Kodama, Y. and Bock, F. G. (1970) Benzo[*a*]pyrene-metabolizing enzyme activity of livers of various strains of mice. *Cancer Res.*, **30**, 1846–1849.
 - 17) Kouri, R. E., Salerno, R. A. and Whitmire, C. E. (1973) Relationships between aryl hydrocarbon hydroxylase inducibility and sensitivity to chemically induced subcutaneous sarcomas in various strains of mice. *J. Natl. Cancer Inst.*, **50**, 363–368.
 - 18) Kouri, R. E., Rattie, H. and Whitmire, C. E. (1973) Evidence of a genetic relationship between susceptibility to 3-methylcholanthrene-induced subcutaneous tumors and inducibility of aryl hydrocarbon hydroxylase. *J. Natl. Cancer Inst.*, **51**, 197–200.
 - 19) Berenblum, I. (1941) The mechanism of carcinogenesis: A study of the significance of cocarcinogenic action and related phenomena. *Cancer Res.*, **1**, 807–814.
 - 20) Singh, N. P., McCoy, M. T., Tice, R. R. and Schneider, E. L. (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.*, **175**, 184–191.
 - 21) Singh, N. P., Tice, R. R., Stephens, R. E. and Schneider, E. L. (1991) A microgel electrophoresis technique for the direct quantitation of DNA damage and repair in individual fibroblasts cultured on microscope slides. *Mutation Res.*, **252**, 289–296.
 - 22) Sato, S., Kitajima, H., Konishi, S., Takizawa, H. and Inui N. (1987) Mouse strain differences in the induction of micronuclei by polycyclic aromatic hydrocarbons. *Mutation Res.*, **192**, 185–189.
 - 23) Sato, S. and Tomita, I. (1998) Response differences among mouse strains in DNA damage and skin carcinogenicity of 7,12-Dimethylbenz[*a*]anthracene are due to inducible aryl hydrocarbon hydroxylase activity. *Biol. Pharm. Bull.*, **21**, 90–92.
 - 24) International Agency for Research on Cancer (IARC) (1987) IARC monographs on the evaluation of carcinogenic risks to humans, *Supplement 7*, Overall Evaluation of Carcinogenicity: An updating of IARC monographs from Vols. 1 to 42, IARC, Lyon.
 - 25) Morita, T., Asano, N., Awogi, T., Sasaki, Y. F., Sato, S., Shimada, H., Sutou, S., Suzuki, T., Wakata, A., Sofuni, T. and Hayashi, M. (1997) Evaluation of the rodent micronucleus assay in the screening of IARC carcinogens (groups 1, 2A and 2B): The summary report of the 6th collaborative study by CSGMT/JEMS.MMS. *Mutation Res.*, **389**, 3–122.
 - 26) MacGregor, J. T., Wehr, C. M. and Gould, D. H. (1980) Clastogen-induced micronuclei in peripheral blood erythrocytes: The basis of an improved micronucleus test. *Environ. Mutagen.*, **2**, 509–514.
 - 27) 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 Res.*, **245**, 245–249.
 - 28) CSGMT (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.
 - 29) Hayashi, M., Kodama, Y., Awogi, T., Suzuki, T., Asita, A. O. and Sofuni T. (1992) The micronucleus assay using peripheral blood reticulocytes from mitomycin C- and cyclophosphamide-treated rats. *Mutation Res.*, **278**, 209–213.
 - 30) Sato, S., Taketomi, M. and Morita, T. (1992) Simplified mouse peripheral reticulocyte micronucleus test with dimethylnitrosamine. *Mutation Res.*, **278**, 103–107.
 - 31) Sato, S. and Taketomi, M. (1994) The mouse micronucleus test with formaldehyde. *MMS Com.*, **2**, 69–73.
 - 32) Tanisho, T., Yagi, T., Iwasaki, K., Shimoji, K., Kinae, N., Hayashi, M. and Sofuni, T. (1998) Monitoring of coastal water contaminated with mutagens and/or carcinogens using micronucleus test in fish. *Environ. Mutagen Res.*, **20**, 1–9.
 - 33) Hayashi, M., Ueda, T., Uyeno, K., Wada, K., Kinae, N., Saotome, K., Tanaka, N., Takai, A., Sasaki, Y. F., Asano, N., Sofuni, T. and Ojima, Y. (1998) Development of genotoxicity assay systems that use aquatic organisms. *Mutation Res.*, **399**, 125–133.
 - 34) Peace, B. E. and Succop, P. (1999) Spontaneous micronucleus frequency and age: What are normal values? *Mutation Res.*, **425**, 225–230.
 - 35) Saotome, K., Sofuni, T. and Hayashi, M. (1999) A micronucleus assay in sea urchin embryos. *Mutation Res.*, **446**, 121–127.
 - 36) Asanami, S., Shimono, K. and Uejima, M. (1993) Micronucleus test in rats by repeated dosing. *Japanese J. of Mutagenicity Tests on Chemicals*, **2**, 29–38.
 - 37) Sato, S., Taketomi, M., Nakajima, M., Kitazawa, M., Shimada, H., Itoh, S., Igarashi, M., Higashikuni, N., Sutou, S., Sasaki, Y. F., Hayashi, M., Sofuni, T., Higashiguti, T., Nito, S., Kondo, Y., Honda, S.,

- Hayashi, M., Shinagawa, Y., Nakajima, E., Oka, Y., Shimoi, K., Hokabe, Y., Morita, A., Kinae, N., Takeuchi, M., Hirono, H., Yamamura, E. and Tamai, K. (1995) Effect of aging on spontaneous micronucleus frequencies in peripheral blood of nine mouse strains: The results of the 7th collaborative study organized by CSGMT/JEMS.MMS. *Mutation Res.*, **338**, 51–57.
- 38) Hamada, S., Sutou, S., Asanami, S., Hosoya, S., Ozawa, S., Kondo, K., Nakajima, M., Shimada, H., Ohsawa, K., Kondo, Y., Asano, N., Morita, T., Sato, S., Tamura, H., Wakata, A., Yajima, N., Marshall, R., Weaver, J. L., Blakey, D. H., Torus, D., Moore, C., Proudlock, R. and Hayashi, M. (1998) Abstract of papers, the 27th Annual Meeting of the Environmental Mutagen Society of Japan, Osaka, p. 78, Nov.
- 39) Gossen, J. A., de Leeuw, W. J. F., Tan, C. H., Zwarthoff, E. C., Berends, F., Lohman, P. H. M., Knook, D. L. and Vijg, J. (1989) Efficient rescue of integrated shuttle vectors from *transgenic mice*: A model for studying mutations *in vivo*. *Proc. Natl. Acad. Sci. (U.S.A.)*, **86**, 7971–7975.
- 40) Kohler, S. W., Provost, G. S., Fieck, A., Kretz, P. L., Bullock, W. O., Sorge, J. A., Putman, D. L. and Short, J. M. (1991) Spectra of spontaneous and mutagen-induced mutations in the *lac I* gene in *transgenic mice*. *Proc. Natl. Acad. Sci. (U.S.A.)*, **88**, 7958–7962.
- 41) Igarashi, M. and Shimada, H. (1997) An improved method for the mouse liver micronucleus test. *Mutation Res.*, **391**, 49–55.
- 42) Nishikawa, T., Haresaku, M., Adachi, K., Masuda, M. and Hayashi, M. (1999) Study of a rat skin *in vivo* micronucleus test: Data generated by mitomycin C and methyl methanesulfonate. *Mutation Res.*, **444**, 159–166.
- 43) Ohshima, W. and Tokumitsu, T. (1996) An *in vivo* micronucleus test using colonic epithelial cells of mice. *Environ. Mut. Res. Commun.*, **17**, 265–269.
- 44) Schröck, E., du Manoir, S., Veldman, T., Schoell, B., Wienberg, J., Ferguson-Smith, M. A., Ning, Y., Ledbetter, D. H., Bar-Am, I., Soenksen, D., Garini, Y. and Ried, T. (1996) Multicolor spectral karyotyping of human chromosomes. *Science*, **273**, 494–497.
- 45) Asano, N., Katsuma, Y., Tamura, H., Higashikuni, N. and Hayashi, M. (1998) An automated new technique for scoring the rodent micronucleus assay: Computerized image analysis of acridine orange supravivally stained peripheral blood cells. *Mutation Res.*, **404**, 149–154.
- 46) Grawé, J., Nüsse, M. and Adler, I-D. (1997) Quantitative and qualitative studies of micronucleus induction in mouse erythrocytes using flow cytometry. I. Measurement of micronucleus induction in peripheral blood polychromatic erythrocytes by chemicals with known and suspected genotoxicity. *Mutagenesis*, **12**, 1–8.
- 47) Grawé, J., Adler, I-D. and Nüsse, M. (1997) Quantitative and qualitative studies of micronucleus induction in mouse erythrocytes using flow cytometry. II. Analysis of micronuclei of aneugenic and clastogenic origin by dual-colour FISH on populations of bone marrow PCEs flow sorted on the basis of their relative DNA content. *Mutagenesis*, **12**, 9–15.
- 48) Dertinger, S. D., Torous, D. K. and Tometsko, K. R. (1997) Flow cytometric analysis of micronucleated reticulocytes in mouse bone marrow. *Mutation Res.*, **390**, 257–262.