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

Sei-ichi Sato* and Isao Tomita

*Japan Tobacco Inc., Toxicology Research Laboratories, Central Pharmaceutical Research Institute, 23 Nakogi, Hatano, Kanagawa 257–0024, Japan and Laboratory of Life Science, Shizuoka Sangyo University, 4–1–1 Surugadai, Fujieda, Shizuoka, 426–8668, Japan

(Received September 8, 2000)

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 assay6–8) 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...
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)\textsuperscript{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.\textsuperscript{9} Recently, a molecular cytogenetic method, \textit{i.e.}, “fluorescent in situ hybridization (FISH)”,\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 demonstrated 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.\textsuperscript{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 \textit{in vivo}.

The current status of the micronucleus assay will be discussed further for its usefulness and limitations.
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) assay20,21) 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, respectively.

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. 2. Formation of Micronucleated Erythrocytes by Mutagens](image)

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.
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. 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 assay 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. The peripheral micronucleus assay can be carried out as shown in Fig. 4. 1) AO solution is spread homogeneously on the slide.
5 µl 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. 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). 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.

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.

Recently, a new gene mutation assay in vivo was developed using transgenic animals, such as, Muta™Mouse or Big Blue™. 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 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, skin and colon epithelium, have recently been employed in addition to the bone marrow micronucleus assay. The labeling of all chromosomes by spectral karyotyping in the FISH method, mentioned in section 1, will be possible using some DNA-probes.

Examination of micronuclei by the human eye is time-consuming. Image analysis scoring or flow cytometry 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


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.,


