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Integration of micronucleus tests with a gene mutation assay in F344 *gpt* delta transgenic rats using benzo[*a*]pyrene

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ABSTRACT

Reduction of the number of animals used in *in vivo* genotoxicity tests is encouraged. For this purpose, we conducted integrated toxicity tests combining gene mutation assays with multiple-organ micronucleus (MN) tests (peripheral blood, bone marrow, liver, and colon) in F344 *gpt* delta transgenic (Tg) rats. Seven-week-old male F344 *gpt* delta rats were orally administered 62.5 or 125 mg/kg/day benzo[*a*]pyrene (B[*a*]P) for 28 days. One day after the final day of treatment (day 29) and three days after the final treatment (day 31), bone marrow, liver, and colon samples were collected, and mutation assays and MN tests were performed. The *gpt* mutant frequency (MF) significantly increased in bone marrow, liver and colon but MN induction was only significant in bone marrow but not in liver and colon. Similarly MN induction was only observed in bone marrow in non-Tg F344 rats. In peripheral blood obtained on day 4, 15, 29, 31, a time-dependent increase was observed in reticulocyte MN frequency during the treatment. Thus, our integrated method successfully detected both gene mutations and MN induction caused by B[*a*]P. In addition, no significant differences were observed between sampling times (day 29 *versus* 31), suggesting that sampling on day 29 is also valid to evaluate gene mutations. On the other hand, MN results in bone marrow and peripheral blood were different depending on the sampling day. An appropriate sampling day should be designated according to which assays are integrated. We confirmed that integration of the MN test with a gene mutation assay using F344 *gpt* delta Tg rats is useful to evaluate different endpoints related to genotoxicity using the same animals and to reduce animal use.

1. Introduction

Conventional safety evaluations of chemical compounds, including pharmaceuticals, are performed on the basis of toxicity tests using experimental animals. Growing concern for animal welfare in recent years has required improvements in evaluations using animals from the perspective of replacement, reduction, and refinement (the “3Rs”). In genotoxicity testing, the 3Rs concept was also considered and reflected in the 2011 revision of the ICH S2 (R1) Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use (2011) [1]. One approach for reducing the number of animals used for testing is to refine *in vitro* genotoxicity test methods, which yield results that can be extrapolated to humans. However, *in vivo* testing is still believed to be important because it enables one to consider the pharmacokinetics of chemical compounds. It is therefore important to integrate multiple evaluation endpoints from a single animal, which will allow reduction of the number of animals required for an *in vivo* test.

Transgenic (Tg) animals created by introducing reporter genes have been widely used for the efficient detection and analysis of mutations [2–4]. Genotoxicity tests using Tg animals are the only way to assess gene mutations in multiple organs. Thus, the combination of a gene mutation assay in Tg animals with other endpoints would facilitate further usage of the gene mutation test for integrated genotoxicity testing. Integration of a gene mutation assay using Tg animals with other genotoxicity tests or repeated-dose toxicity test has been reported [5–9], but little consideration has been given to the sampling timing of organs that allows suitable evaluation by each of the integrated assays.

The *in vivo* micronucleus (MN) test using bone marrow is performed to assess the clastogenicity/aneugenicity of chemicals and is included in

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many genotoxic test guidelines for multiple classes of chemicals. An *in vivo* MN test using peripheral blood has also been established, and results after 28 days of repeated administration have been confirmed [10,11]. The incorporation of *in vivo* erythrocyte MN tests to general toxicity tests has recently been suggested as a means of promoting the 3Rs [1,12,13]. To this end, *in vivo* micronucleus tests in multiple organs, in addition to bone marrow and peripheral blood, have been developed for conventional use. Recent investigations have focused on MN tests for liver, an important site of metabolism, and the gastrointestinal tract, the first site of direct contact with ingested chemicals. Reports of MN tests that allow detection of MN induction by typical genotoxic carcinogens that target these organs, and the integration of these tests with general toxicity tests, have been published [14,15].

In this study, we employed a mutation assay using F344 *gpt* delta Tg rats and combined it with multiple-organ MN tests with the aim of establishing an integrated genotoxicity test method. *gpt* delta rodents, developed previously [3,16,17], have the reporter gene λEG10 in their genome to allow the detection of mutations. Gene mutation assays and MN tests for bone marrow, liver, and colon, as well as MN test for peripheral blood, were performed using tissues from the same animals. The test compound used in this study was benzo[*a*]pyrene (B[*a*]P), a well-characterized genotoxic carcinogen for rodents for which much toxicological data is available [18–21].

We also investigated the effect of tissue sampling time. The OECD Guideline for Tg assays (TG488) recommends repeated oral administration of the test compound for 28 days, with tissue sampling three days after the final administration (28 + 3) [22]. However, this specificity in sampling time is inconvenient for integration with other evaluation techniques that detect other endpoints. We therefore compared the 28 + 3 protocol, which is in accordance with the Tg assay Guideline, and the standard protocol (28 + 1) for general toxicity tests, where sampling is done one day after the final administration.

2. Materials and methods

2.1. Chemical

Benzo[*a*]pyrene (B[*a*]P; CAS No. 50-32-8) was purchased from Sigma-Aldrich (Tokyo, Japan) and dissolved in olive oil.

2.2. Animals

Male F344 *gpt* delta transgenic rats and male F344 rats were purchased from Japan SLC, Inc. (Shizuoka, Japan) at 7 weeks of age and used after a 5–7 day acclimation period. The animal room conditions were maintained at a temperature of 23.5 ± 2.0 °C, a relative humidity of 55 ± 5%, a light-dark cycle of 12:12 h, and 12–15 air changes per h. The rats were housed individually (*gpt* delta rats) or with 2–3 animals per plastic cage (non-Tg rats) with wood-chip bedding (Iwakura, Hokkaido, Japan) and free access to tap water and a CE-2 pellet diet (CLEA Japan Inc., Tokyo, Japan). All protocols for animal procedures were approved by the Ethics Committee of Animal Experiment in accordance with the Internal Regulations on Animal Experiments at Suntory, which are based on the Law for the Humane Treatment and Management of Animals (Law No. 105, 1 October 1973, as amended on 30 May 2014).

2.3. Integration of the MN test and gene mutation assay using F344 gpt delta transgenic rats

2.3.1. Treatment of gpt delta rats

Five *gpt* delta rats per group were dosed with B[*a*]P at 0 (olive oil), 62.5, or 125 mg/kg by oral gavage, once daily for 28 days (days 1–28). All dose volumes were set at 10 ml/kg/day. The highest dose level of B[*a*]P was set at 125 mg/kg as this is the anticipated maximum tolerated dose (MTD), according to the results of 28-day repeated administration tests reported in previous papers [11,23,24]. Body weight was measured daily from the first day of administration to the day of autopsy.

2.3.2. Sampling of tissue and blood

One day after (day 29) and 3 days after (day 31) the last day of administration (day 28), blood was collected from the abdominal aorta of anesthetized animals, and then organs were removed. The liver and spleen were weighed. Cells for MN tests were collected from a portion of the bone marrow (femur), liver, and colon immediately after extraction, while separate portions were frozen and preserved at −80 °C until use in mutation assays. Remaining liver, colon, spleen, and sternal marrow were fixed in 10% neutral buffer formalin for use in histopathological examination.

Blood for use in peripheral blood MN tests was collected 1 day before administration (day 0), and on days 4, 15, 29, and 31. Blood was sampled from day 4 and thereafter 24 h after the previous day's administration. Caudal veins were used for blood sampling except on the day of dissection. A portion of the blood taken at the time of dissection was used for peripheral blood MN testing, while the remainder was used for hematological and blood chemistry testing. Hematological testing was performed using a Sysmex XT-1800i analyzer (Sysmex, Milton Keynes, UK).

2.3.3. gpt assays

The *gpt* assay was conducted in accordance with previously published methods [3,25,26]. Mutant frequency (MF) was measured in the *gpt* assay by using 6-thioguanine (6-TG) selection. Genomic DNA was extracted from each tissue using a RecoverEase DNA Isolation Kit (Agilent Technologies, Santa Clara, CA), and λEG10 DNA was recovered as λphage through *in vitro* packaging using Transpack Packaging Extract (Agilent Technologies). Phages were infected into Cre recombinase-expressing *Escherichia coli* YG6020 strain, and the infected strain was incubated at 37 °C in M9 agar culture medium containing chloramphenicol with or without 6-TG. The number of mutant colonies carrying mutant *gpt* genes, which grew on plates with 6-TG, were counted. The *gpt* MF value was calculated by dividing the number of mutant colonies by the number of colonies that grew on plates without 6-TG.

2.3.4. Bone marrow MN test

As reported previously [11,27], bone marrow cells recovered from femurs were suspended in fetal bovine serum (FBS) and smeared onto a glass slide. Cells were stained with acridine orange (AO), and micronucleated (MNed) cells were counted using a fluorescence microscope. Two thousand reticulocytes (polychromatic erythrocytes, PCE) per specimen and the number of PCE per 500 erythrocytes per specimen were observed according to TG474 at the time of study [28].

2.3.5. Liver MN test

As reported previously [11], approximately 1 g of liver was collected and incubated for 1 h at 37 °C in collagenase solution (100 U/mL, pH 7.5). Separated cells were fixed in 10% neutral formalin solution and refrigerated until observation. This liver cell suspension was stained with AO and 4′, 6-diamidino-2-phenylindole (DAPI) solution and observed using a fluorescence microscope. The number of MNed cells was determined in a population of 2000 cells per specimen, and the number of metaphase cells was recorded.

2.3.6. Colon MN test

As reported previously [29], colon samples were incubated for 30 min at 35 °C in 1 mM ethylenediaminetetraacetic acid (EDTA)-Hank's Balanced Salt Solution (HBSS) to recover epithelial cells, which were fixed in 10% neutral formalin solution. The obtained cell suspension was stained with AO and DAPI solution and observed using a fluorescence microscope. The number of MNed cells was determined in a population of 2000 cells per specimen.