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Flow cytometric analysis of micronuclei in rat peripheral blood: An interlaboratory reproducibility study

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ABSTRACT

In anticipation of proposed OECD guideline changes that may include increasing the number of reticulocytes scored for micronuclei, an inter-laboratory reproducibility study of the rat peripheral blood micronucleus assay was performed using flow cytometry. In this experiment, male Sprague–Dawley (SD) rats were treated with the model clastogen cyclophosphamide (CP: 5, 10 or 15 mg/kg) by a single oral administration. As controls, rats were treated with physiological saline (solvent) in the same manner as for the model clastogen. Peripheral blood was collected from each rat 48 h after the treatment. The blood samples were prepared at the Public Interest Incorporated Foundation, BioSafety Research Center (BSRC) in duplicate using the rat MicroFlow^{PLUS} Kit. After fixation, one replicate set of samples was shipped to Litron Laboratories, and each sample was analyzed by flow cytometry at the two laboratories. In addition, the frequency of micronucleated reticulocytes (MNRETs) was determined at the BSRC by microscopic analysis using supravital acridine orange (AO) staining. The reproducibility of micronucleated reticulocyte frequencies analyzed by microscopy and flow cytometry showed good correlation ($r^2 = 0.84$). The frequencies of micronucleated reticulocytes analyzed by flow cytometry at the two independent laboratories showed good concordance ($r^2 = 0.97$). The data indicate that the flow cytometric micronucleus analysis method is a good alternative to manual microscopic analysis. Flow cytometry allows groups to readily score 5000 or even 20,000 RETs in a matter of minutes compared to manual analysis. This results in increased reliability of the assay by achieving better statistical power.

1. Introduction

The *in vivo* rodent erythrocyte micronucleus test is widely and frequently used in regulatory assessment to evaluate the potential of chemicals to cause chromosomal damage. Traditionally, this test has been conducted in mice or rats by the microscopic analysis of MNRETs in bone marrow samples obtained at appropriate times following treatment [1,2]. In mice, the use of peripheral blood for the micronucleus test has been acceptable along with the standard use of bone marrow [3]. In the past, concern over selective splenic removal of micronucleated red blood cells from the peripheral circulation limited the use of blood measurements in rats [4]. However, rat blood is now accepted for use for micronucleus analysis if scoring focuses on the newly formed (younger) reticulocytes and if a sufficient number of cells are scored [5,6]. Flow cytometric scoring methods satisfy these requirements, and provide obvious advantages over manual scoring, *i.e.* shorter analysis time, larger number of cells evaluated, objective *versus* subjective analysis resulting in reduction of scoring error, and the potential for integration into other toxicology studies (*e.g.*, acute and sub-chronic toxicology and pharmacokinetic studies). Litron Laboratories developed a flow cytometric method based on anti-CD71-FITC staining to identify young reticulocytes (commercially available as "MicroFlow^{PLUS}® Kit [7,8]). In this method, accurate and reliable flow cytometer set-up is achieved using a malaria biostandard—that is, fixed erythrocytes from rodents that had been infected with *Plasmodium berghei* [9,10]. To confirm the utility and reproducibility of this method, an interlaboratory study was performed at BSRC and Litron using different models of flow cytometer. In addition, the data from the flow cytometric method was compared with that of microscopic scoring using AO staining to confirm the reliability of the data.

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2. Materials and methods

2.1. Reagents

Cyclophosphamide (CP: CAS No. 50-18-0) was purchased from Shionogi & Co., Ltd. (Osaka, Japan), and was dissolved in physiological saline (Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) immediately prior to use. MicroFlow^{PLUS} Kit was purchased from Litron Laboratories (NY, USA). The kit contained: anticoagulant solution, buffer solution, long term storage solution, RNase solution, anti-CD71 antibody (anti-CD71-FITC), DNA stain, platelet antibody (anti-CD61-PE), and biological standards (*Plasmodium berghei*-infected rat erythrocytes; malaria biostandard) for calibration of the flow cytometer.

2.2. Animals

Male Crl:CD (SD) rats, 6 weeks old, were purchased from Charles River Japan Inc. (Atsugi, Japan). The rats were housed in a negative-pressure air-conditioned unit (22–27 °C, 35–75% relative humidity) for animal housing on a 12:12-h light/dark cycle. After 7-day acclimation, the rats were randomly assigned to study groups. Standard rodent pellet diet (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) was fed and drinking water was available *ad libitum*. The animal experiment was conducted in compliance with the Law Concerning the Protection and Control of Animals of Japan and the Relief of Pain and Guidelines for Animal Experimentation (BSRC Institutional Animal Care and Use Committee Authorization).

2.3. Treatment protocol

CP was orally administered once to 5 male rats per group at dose levels of 5, 10 and 15 mg/kg. The dose level was selected based on previous papers [6,11]. The dose volume administered was 1.0 mL per 100 g of body weight. As a negative control, physiological saline (vehicle) was orally administered in a similar manner.

2.4. Blood sample collection, processing and flow cytometric analysis

Forty-eight hours after the administration, approximately 100 μL of peripheral blood was collected from the tail vein into a syringe containing 350 μL of anticoagulant. After mixing blood and anticoagulant, 180 μL were fixed in duplicate using procedures described in the MicroFlow^{PLUS} Kit manual. Briefly, blood samples were fixed into 2 mL of ultracold (−80 °C) methanol and were then stored at −80 °C. All samples were washed out of fixative with 12 mL of ice-cold buffer solution. One sample per animal was transferred into 1 mL of long-term storage solution, stored at −80 °C and then shipped to Litron for flow cytometric analysis. The replicate washed samples were labeled and analyzed at BSRC as follows: 20 μL of each washed sample was mixed with 80 μL of labeling solution containing RNase solution, anti-CD71 antibody and platelet antibody. The mixed samples were protected from light and refrigerated for 30 min. Next, the samples were incubated at room temperature for 30 min to ensure complete degradation of cellular RNA. After the incubation, the samples were stored in a refrigerator until analysis.

Before analyzing samples, a malaria biostandard sample was used for flow cytometer setup and calibration as described in the kit manual. Each sample was suspended with 1–2 mL DNA staining solution and analyzed by a Cytomics FC500 flow cytometer (Beckman Coulter, CA, USA) at BSRC or by a FACSCalibur flow cytometer (Becton Dickinson, CA, USA) at Litron Laboratories. Anti-CD71 antibodies distinguished mature erythrocytes (CD71-negative) from RETs (CD71-positive), while PI was used to detect cells with DNA. Platelet antibodies were used to detect and remove platelets from the analysis. Therefore, the population of interest, MNRET, was identified as those cells having high levels of both CD71 and PI-associated fluorescence. The stop mode was set so that approximately 20,000 CD71-positive erythrocytes (RETs) were analyzed per blood sample.

2.5. Microscopic analysis (AO supravital staining method)

The AO supravital staining for MNRET was performed according to the method of Hayashi et al. [3,12]. Forty-eight hours after the administration, approximately 8 μL of peripheral blood was obtained directly from the tail vein and mixed with 8 μL of fetal bovine serum. Approximately 7 μL of the mixed blood was dropped on a coverslip, and immediately covered with an AO-coated slide. Slides were sealed with Permount and refrigerated until analysis. Two thousand RETs were analyzed per animal using a fluorescent microscope (800×) equipped with a blue excitation filter, and the number of MNRETs was scored. In addition, the number of RETs was scored by examining 500 erythrocytes (RETs + mature cells) per animal to estimate cytotoxicity.

3. Results and discussion

In addition to the sensitivity of the micronucleus assay, the specificity and feasibility for hazard identification are also important. One of the simplest ways to increase sensitivity is to increase the sample size, namely the number of cells analyzed. However, it is resource intensive to increase the number of cells analyzed using microscopy. A significant advantage to using flow cytometry is the ability to greatly increase the number of cells scored without depleting resources. Prior to using the flow cytometric micronucleus assay as a routine method, it should be validated in-house by demonstrating concordance of the data with comparative data obtained by microscopy. The reproducibility of the data obtained at different laboratories using different flow cytometers can also be evaluated.

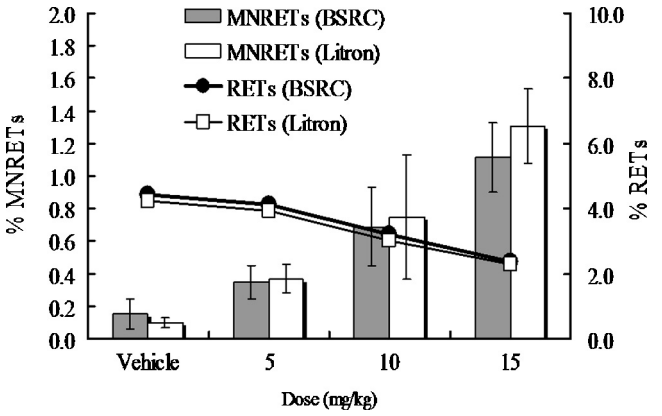

Fig. 1. Frequencies of micronucleated reticulocytes (%MNRETs) and reticulocytes (%RETs) in peripheral blood of rats after single oral administration with CP at 5, 10 and 15 mg/kg. Blood was collected at 48 h after the administration. Each group consisted of 5 rats. These data were acquired by flow cytometric analysis at BSRC and Litron Laboratories.

To show concordance and reproducibility, we used the well known clastogen CP as a model chemical. Blood samples were collected, and the frequency of MNRETs (%MNRETs) and the frequency of RETs (%RETs) were analyzed by flow cytometry at the two laboratories (BSRC and Litron) independently. Results are shown in Fig. 1. Dose–response curves of %MNRET and %RET were almost identical between the two laboratories. The correlation of %MNRET and %RET between BSRC and Litron was analyzed by linear regression and showed a good correspondence (both %MNRET and %RET: $r^2 = 0.97$, Fig. 2), although the flow cytometer used at each laboratory was a different model.

To evaluate the concordance of data obtained manually and flow cytometrically, MN were scored by microscopy using AO supravital staining at BSRC, and the data were compared to those obtained by flow cytometry. Results are shown in Fig. 3. Dose–response curves of %MNRET were similar for data obtained by both methods ($r^2 = 0.84$, Fig. 4). The %RET obtained using microscopic analysis tended to be higher than those by flow cytometric analysis. The correlation analysis shows a lower correlation ($r^2 = 0.63$) compared to that of %MNRET. This phenomenon may be explained by different thresholds in the age cohort of RETs analyzed by each method, *i.e.* AO supravital staining, where the analysis is restricted to RET with the a high content of RNA, and the flow cytometric method, where the analysis is restricted to a subpopulation of high RNA containing RET based on CD71 expression. Although the %RET was observed to be lower by flow compared to microscopy, the ability to score tens of thousands of RETs per sample enhances the precision of the MNRET measurements. This reduces the likelihood of missing weakly genotoxic agents.

Based on the results of this study, high reproducibility of data between laboratories was confirmed, and an advantage of the flow cytometric scoring method was recognized. MacGregor et al. [6] reported that inter- and intra-laboratory reproducibility is higher by flow cytometry (peripheral blood) than manual microscopy (bone marrow). The higher analytical performance of the automated flow cytometric scoring method in the MN assay is the